

Human rights in China

MANY Western scientists have experienced the excitement in the past few years of establishing or re-establishing ties with scientists from the People's Republic of China. The openness and frankness of these exchanges (particularly in comparison with the unreal nature of some exchanges with the Soviet Union) has surprised and pleased those involved. Even though there clearly have been and continue to be strong political winds blowing in China, it has been widely anticipated that China will continue to take a fuller place in the international scientific community. But many of the more thoughtful visitors have left wondering what the true price is that the people have to pay to live in modern China. Some inkling of this now emerges from a recent Amnesty International report 'Political Imprisonment in the People's Republic of China'.

The constitution guarantees a number of basic rights, such as 'freedom of speech, correspondence, the press assembly, association, procession, demonstration and the freedom to strike'. In addition citizens have the right to 'speak out freely, air their views fully, hold great debates and write big-character posters'. Furthermore the state 'applies the principle of "letting a hundred flowers blossom and a hundred schools of thought contend" so as to promote the development of the arts and sciences and bring about a flourishing socialist culture'. However, all cultural undertakings must

'serve the workers, peasants and soldiers and serve socialism', and the state 'suppresses all treasonable and counter-revolutionary activities, punishes all traitors and counter-revolutionaries, and punishes newborn bourgeois elements' and 'other bad elements'.

A continuous campaign of purification seems to go on against counter-revolutionary elements, who face a wide range of punishments from public criticism to execution for their crimes of announcing reactionary solutions or writing reactionary posters. Trials leave a lot to be desired; the main aim seems to be to extract a confession, and defence is a rare luxury. Indeed those who refuse to acknowledge their wrongdoing are subjected to much severer sentences. The number of counter-revolutionaries that courts have dealt with is difficult to estimate, but clearly runs into many tens of thousands. Chairman Mao himself once estimated the hardcore of reactionaries at two per cent of the whole population.

The Amnesty report is not cheerful reading; the crude ways in which those who do not follow the official line are humiliated, imprisoned or even exterminated cannot but worry all those who wish to establish the closer diplomatic, cultural and intellectual relations with China. And presumably for every one who runs foul of the judicial system for unacceptable views there are many who simply choose to remain silent. □

Research councils dislocated

ON December 18, Shirley Williams, Britain's Secretary of State for Education and Science will officially open the new premises of the Science Research Council and the Natural Environment Research Council at Swindon. This is the final step in a five-year process by which all but a handful of the staff of the two councils have been moved out of London. And the true dimensions of the inconvenience it will cause are now becoming all too clear.

For many of the staff, of course, the move to a more rural environment has had its attractions. Those whose work does not directly involve attending or servicing meetings or being on hand for the scientist who wishes to drop in may well feel that they have gained. But those whose effectiveness depends on easy contact with a wide variety of people have much more reason to complain. In the SRC, for instance, the Chairman's office stays in London, as do the directorates of marine technology and polymer engineering. But the Secretary to the Council is in Swindon, as is the Press Office. Both chairman and secretary are expecting to have to spend

an average of a day and a half per week at the other end of the line.

At present SRC council and committee meetings are continuing to be held in London, scattered around the premises of various learned societies. This is hardly an ideal solution, but in the future, wear and tear on council staff may well lead to pressure for more meetings to be held in Swindon, entailing complex cross-country journeys for those who do not live near London, and presumably necessitating more overnight stays. Fine in some respects; if visiting academics and industrialists can be prevailed upon to give up an extra half day, the quality of committee work will probably rise. But it is every bit as likely that attendance at meetings will fall.

Life in Swindon will be fine for a majority of the councils' staff. But the more senior the person, the more inconvenient and tiring it will be. The net result could well be that it will become much more difficult to recruit to the top posts, and that committee members will become much less willing to give of their time. □



Crisis for sperm whales

The International Whaling Commission (IWC) is holding a special meeting in Japan next week. Two major tasks for the meeting will be to set catch limits for sperm whale stocks in the North Pacific and to review the status and catch limits of sperm whales in the Southern Hemisphere. John Beddington, lecturer in population biology at the University of York, explains why he thinks no more sperm whales should be caught in these areas.

AT ITS meeting in Cambridge this summer the scientific committee of the International Whaling Commission identified a number of worrying questions concerning the current state of the world's sperm whale stocks. These questions, which were the subject of further discussions when the committee reconvened in La Jolla in November, pose problems both for the whales and the IWC.

Since the 19th century the sperm whalers have concentrated their hunt on the older males, as there are sound economic reasons for preferring these larger animals. More recently it has been recognised that because the sperm whale is polygamous there is a clear opportunity of obtaining an increased yield from populations with a substantially disturbed sex ratio. However, there is a danger that if too many mature males are taken, pregnancy rates will fall as females fail to find mates. This danger is recognised in the current management model used by the IWC which demands that a reserve of socially mature males should be excluded from exploitation to ensure maximal pregnancy. Unfortunately, in two major sperm whale stocks there have been disturbing falls in the pregnancy rates of the female catch. These changes, in the Australian sector of the Southern Ocean and in the Western division of the North Pacific gave a clear indication that all was not well with these stocks.

This problem was illuminated by other scientific analysis by Beddington, Holt, and Fowler which showed that the way in which the IWC had estimated population abundance seriously masked the extent of the depletion of stocks following exploitation. The mechanism involved is simple. The main measure of population abundance used by the IWC is the catch per unit of fishing effort and the measure of effort that has been used was the number of catcher boat days. However, catching whales takes time and daily catch rates at the start of the fishery were limited not by the population abundance but by the time taken for catching whales. Accordingly as stocks declined there was no corresponding drop in catch rates and hence stocks were thought to be more abundant than was in fact the case.

When data for the Western North Pacific stock were reanalysed in the light of these observations it was found that in the period of the observed fall in pregnancy rates the mature male population had fallen below the reserve level and hence pregnancy rates would have been expected to fall. Similar analysis of the Australian stock was not carried out in Cambridge due to lack of time, but some members of the Scientific Committee were of the view that given the substantial fall in pregnancy rates, a similar effect was occurring and that to set a zero quota would be the appropriate response of the commission. In the event this view was not accepted and the quota for this division was reduced by only 25%. Unfortunately, subsequent analysis submitted to the Australian Whaling Enquiry by Kirkwood, Allen & Bannister vindicates this pessimistic view and indicates that here again mature males were reduced below their reserve level and that pregnancy rates

would be expected to have fallen. The status of both these stocks gives much cause for concern and it should be noted that as comparable data on the pregnancy rates are not available for all stocks the problem may not be restricted to these areas alone.

A final problem highlighted by the sperm whale analysis concerns the inadequacies of the new management procedure used by the IWC to set quotas. This procedure provides for protection of stocks when they fall 10% below their maximum sustainable yield level. In both the cases considered above, strict interpretation of these rules would permit quotas to be taken—females from the Australian division of the Southern Ocean and both sexes from the Western North Pacific stock. However, simple extrapolation of the demographic statistics indicate that even in the absence of exploitation these stocks would continue to decline for decades. If subsequent analysis corroborates these findings the Whaling Commission will thus be faced with the difficult task of deciding whether to interpret its rules legalistically or to protect these whale stocks. □

Cornelia Durrant, Wildlife Campaigner at Friends of the Earth, UK, adds: At the end of last October it seemed that the UK government had taken some action to protect whales when it announced its proposals for the meeting of the Convention on the International Trade in Endangered Species (CITES) to be held next March. CITES aims to control international trade in wildlife and wildlife products. Those species which the 48 member nations agree are 'endangered' are listed on appendix I of the convention and trade in them is normally forbidden. Those which are considered 'threatened' are listed on appendix II. Trade in them is allowed but monitored.

The UK has been proposing a review of cetacea for next year's CITES meeting. It proposes that 11 species of dolphins and porpoises should be put on appendix I and that all cetacea not on appendix I should be listed on appendix II—including the sperm whale.

When this was announced it received a great deal of attention. So much so that the implications of the UK's other proposal to CITES were not fully explained. The convention requires all 'readily recognisable' parts and products of listed species to be controlled, but there is disagreement over what is recognisable, and many members control no products at all. Together with Switzerland and West Germany, the UK has prepared a list of parts and derivatives of several species of animal which it would like to see all parties control.

This list, called the 'minimum list' of parts and products, is surprising in many respects. Most surprising of all it does not include whale products because "whale meat, meat products and oil were not considered to be identifiable without the use of biochemical laboratory equipment". However, the UK has always identified whale products for Customs tariff purposes.

Consequently even if all cetaceans are put on the appendices to CITES only trade in live animals will be controlled—and this is only a few dolphins a year. The trade in their products will continue unchecked.

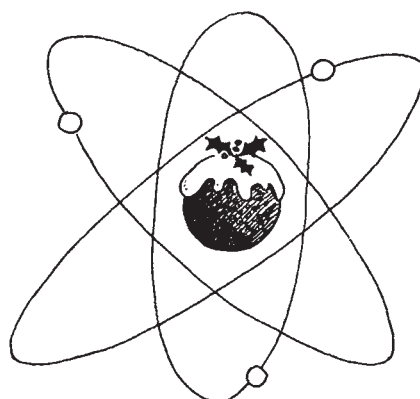
Recent data on the status of sperm whales shows several populations to be more than just 'threatened', in particular those in the Western division of the North Pacific and those in divisions five and seven of the Southern Hemisphere. Consequently there is a strong case for the UK proposing these populations of sperm whales for appendix I of CITES. If, however, the 'minimum list' is accepted in its present form this would be of little use. As it is clearly impossible to distinguish between the products of whales from different populations the only way to ensure that the endangered ones are not further depleted is to control the products of all sperm whales. □

Surprise Christmas present for British science

AN unexpected Christmas bonus for British science was revealed this week by the Secretary of State for Education and Science, Mrs Shirley Williams, who announced that the country's science budget was to be increased by more than £47 million over the next four years. More than 70% of this money is to go to the Science Research Council and the remainder is to be divided between the Agricultural Research Council, the Medical Research Council, and the Natural Environment Research Council. Only the Social Science Research Council budget remains static.

The budget increase represents a significant Cabinet victory for Mrs Williams who has been arguing strongly all year for increases in funds for basic scientific research—which, she said, had suffered too severely at the expense of applied research. Now the science budget is to be increased by £10 million in 1979-80; £10 million in 1980-81; £12 million in 1981-82 and £15 million in 1982-83. The move will increase next year's budget to £274 million of which £149.7 million will go to the SRC.

The increase reflected a change in world opinion which was now swinging back to a more favourable view of basic research, said Sir Frederick Stewart, chairman of the Advisory Board for the Research Councils, the body responsible for dispersing the science budget among the five research councils. He quoted recent developments in the United States and France where there had been increases in



money for fundamental research.

Mrs Williams said Britain had insufficiently recognised the achievements of its basic science which was regarded highly elsewhere in the world. This extra cash would attempt to remedy this and would also be used to create new research positions for able young students who were now suffering severe problems in finding posts.

Predictably the happiest research council chairman was Professor

Geoffrey Allen of the SRC who described himself as "delighted" with his cash allocation. Last month he announced at the publication of his council's annual report that the SRC needed only £20-£30 million to carry out a full but realistic programme of research over the next four years. In fact, the budget increase means he will now have slightly more than £30 million for this programme, of which £4 million will become available next year.

Initially much of this money will go to restoring the SRC's construction programme to previous levels, including the speedy completion of synchrotron radiation, laser and electron beam lithography facilities at the SRC's laboratories, and also in setting up research programmes in computer and microprocessor technology. It is also hoped that Britain will get a new space programme involving participation in the United States' MMS re-usable satellite project.

As for the other research councils, it is likely that the extra cash will be used on projects including increased research into cancer and its treatment and also to set up a new unit of environmental epidemiology for the Medical Research Council; to improve geological work in deep drilling and mapping for the Natural Environment Research Council; and to support work for the Agricultural Council which would involve developing crops which could fix their own atmospheric nitrogen.

Robin McKie

Research allocations

	1979-80	1980-81	1981-82	1982-83
	£m	%	%	%
ARC	24.7	+1.9	+1.1	+1.0
MRC	46.5	+1.6	+0.9	+1.0
NERC	31.1	+2.5	+1.5	+1.0
SRC	149.7	-1.0	-0.5	+1.0
SSRC	14.8	0.0	0.0	0.0
BM(NH)	4.6	+0.5	+0.5	+1.0
Royal Soc	2.6	+1.0	+1.0	+1.0
	<u>274.0</u>			

Blind man's buff at GMAG

ON 31 December, the two-year term of office for members of the UK's Genetic Manipulation Advisory Group (GMAG) will come to an end; some members will be leaving and new people appointed in their place. But the criteria used to decide who will stay on and who will leave are a deepening mystery.

The only official information that has been made public about the changes is that Sir William Henderson (until recently Secretary to the Agricultural Research Council) will succeed Sir Gordon Wolstenholme as chairman. A spokesman at the Department of Education and Science told *Nature* last week that in the interest of "balance and continuity", the four sectors represented on GMAG (science, industry, the unions and the public) would continue to be represented and that not all the members of the existing GMAG would be asked to stand down.

According to John Morris, GMAG secretary at the Medical Research Council, however, that does not mean that "some members who may wish to stay on will not be sacked".

At least one member of the group, Dr J. R. Ravetz, a public interest representative, can testify to this. Three months ago all members of GMAG were sent a letter asking if they wished to continue. Dr Ravetz, a reader in the history and philosophy of science at the University of Leeds, had played a particularly active role in GMAG and did not wish to leave, but was sent a letter asking him to stand down. He was given no reason other than that new appointments "are necessary to ensure the balance and continuity of the group".

However, two of the three other public interest representatives have indicated that they wish to stand down for personal reasons. Continuity in the

public interest sector, Ravetz argues, is therefore being carried on the shoulders of the only remaining public representative, John Maddox. Mr Maddox was unaware that two of the public representatives were leaving voluntarily until contacted by *Nature* last week.

What then are the criteria used to decide which representatives should be removed, and who is responsible for making these changes? For the union and industry representatives, the situation is clear. They are nominated or recommended to stand down by their respective organisations and these recommendations are generally accepted by the DES. The Trades Union Congress (TUC) has asked to be represented on the new GMAG by its four original representatives and by one new member. The four serving TUC members have been reappointed by the DES, but there is no word yet about

the request for a fifth representative. The Confederation of British Industry has requested that its representative Mr John Gilby, be renominated and is awaiting a reply.

The situation of the science and public interest representatives is, however, considerably less clear. They are directly appointed by the DES but there appears to be no set procedure governing these appointments beyond "usefulness to the group". Ravetz has certainly been useful, but he has also not shrunk from expressing his opinions, and he may have been seen by some close to GMAG to be 'rocking the boat'.

According to one DES spokesman, the public interest representatives are the ones most likely to change "because

the scientific specialists are a small group to choose from", but he expects that the overall "mixture of representatives" will remain the same—except that possibly a lawyer will be included on GMAG for the first time. As to who at the DES is responsible for the changes on GMAG, several sources indicate that Shirley Williams, the Education Secretary, is taking a direct and personal interest in the new appointments. However, her advisers on GMAG remain in the shadows and do not appear to include GMAG's existing members.

All this might be unimportant if it were not symptomatic of how GMAG handles its external relations. And external relations are important to a body whose decisions can affect the

competitiveness of British biotechnology industry overseas. In this context, it is obviously desirable to get GMAG's standards accepted and applied by other countries. Unfortunately GMAG does not appear to communicate effectively with foreign scientific organisations. There is a strong feeling among some European scientists that GMAG could provide greater leadership in Europe by revealing more details of its work. As Dr Ravetz argues: "in about a year an American GMAG could be established that would operate in public and be easily accessible to foreign scientists. If that happens, the British GMAG would probably be ignored rather than be viewed as a model for all the world".

A. J. McClelland

Difficulties at PETRA worry designers of Europe's next accelerator

PETRA, the world's biggest storage ring for colliding electrons with positrons, is not behaving quite as expected, writes Konrad Guettler

THE European Committee for Future Accelerators (ECFA) convened its technical panel on the design of LEP—Europe's proposal for a 70 to 100 GeV electron and positron storage ring—in Rome recently, only to hear that the machine of which much LEP design has been based (West Germany's 19 GeV PETRA) is encountering difficulties.

Although PETRA started up very smoothly, ahead of schedule, and soon achieved beams of long lifetime, its luminosity (which determines the rate at which experiments can be done) is at present a factor of 100 or so below design. The profound worry is that the scaling up of parameters from lower energy machines, such as the 10 GeV DORIS and SPEAR, to the very much higher energies of PETRA or LEP may in fact not be straightforward, or indeed possible at all. It is still early days for PETRA but a large investigation program on both the technical and the theoretical side has now been launched both at DESY and by ECFA.

Nicola Cabibbo, a particle theorist at Rome University, and CERN are directing the attempts at increasing theoretical understanding of the observed beam properties. The main effects are the following:

- the maximum beam-beam tune shift ' ΔQ ' is much less than its design value. ΔQ is the major factor, apart from the stored particle current, determining the machine luminosity, ie the number of interactions that take place at an intersection in unit time. The tune

shift is a measure of the non-linear transverse forces between colliding bunches. All the existing machines turned out to have the same limiting value; and this ΔQ had also been assumed for PETRA and for LEP.

- Fast beam instabilities occur at various stored currents and appear to depend on the accelerating radio-frequency voltage. The circulating beams induce currents in the vacuum chamber walls and these wall currents create fields which interact directly with the beam. They can alter the normal betatron and synchrotron frequencies of the beam and thus cause instabilities.

- The accelerated bunches are larger than expected. (This affects the long-term beam stability.) Bunch lengthening is again due to the short-range fields induced by the bunch in the wall. It leads to a wider energy spread within the bunch and can lead to head-tail instabilities.

There was a lot of concern at Rome that there was inadequate knowledge

of beam dynamics at high energies. But a lot of experience is accumulating about high energy electron-positron machines and there was a widespread hope that while machine physicists have encountered very tricky problems indeed, they are unlikely to lead to profound revisions in the approach to LEP.

Since ECFA's Rome meeting, PETRA has run continuously and machine physicists have now pushed the beam current to a maximum of 18mA per bunch, almost up to the 20mA limitation design value. The previous current limitations have been overcome by changing the injection optics to the type also proposed for LEP. The present aim is for fast injection and a high beam intensity, and only later will PETRA go for higher energy, and hopefully, higher luminosity. The latest machine runs at a centre-of-mass energy of 16 GeV have yielded 1-2 hadronic events per nanobarn cross-section per day—which can be compared with 20-100 events at DORIS. This is not a very high luminosity; but the DESY machine physicists are confident about increasing it. Higher energies have to wait till next year when additional accelerating cavities will be switched in. □

Keeping down the cost of LEP

HIGH-ENERGY physicists have become very aware of current financial constraints, and are paying a great deal of attention to reducing the cost of LEP, without diminishing its physics potential. CERN has estimated the construction cost of a 70 GeV LEP to be a little over 1,000 MSF, which is almost the same as the SPS proton accelerator built at CERN a few years ago. No

decision about the project has been taken, but ECFA hopes to present a detailed design study to the CERN Council by the end of 1979.

The physics interest in LEP is focussed on the maximum energy of the machine. Lepton physics at high energies centres around the role of the intermediate vector bosons, the charged W^+ or W^- and the neutral Z^0 .

Their masses can be estimated from the 'Weinberg angle' measured in neutrino interactions. At present predictions of the Z^0 and the W^+ mass are 89 ± 5 GeV and 78 ± 6 GeV, respectively. Single beams of about 45 GeV should be sufficient to produce Z^0 's in association with other particles but the full machine energy will be required to create a W^+W^- pair.

For technical and cost reasons, the original CERN design study (LEP-70, completed this summer) was optimised for an energy of 70 GeV; but it is now being redone for 80-85 GeV per beam. The size and cost of any such machine increase with its maximum energy, and LEP-70 already had a circumference of about 30 km. However, valuable experience was gained from the LEP-70 design exercise. In particular the cost sensitivity of machine parameters and components can now be assessed more reliably.

Wolfgang Schnell, of the CERN ISR division, has already proposed some interesting ways of reducing the construction cost of the future accelerator. LEP will have more than 2,500 dipole bending magnets to keep the particles on their orbits. The bending power of each magnet is low because too high a track curvature would make the electrons lose too much of their energy by synchrotron radiation. Conventionally, magnets are made by precisely stacking hundreds of thin steel plates and welding them together. But the low magnetic fields required for LEP (to reduce bending, synchrotron radiation, and

running costs) can be obtained by bonding a smaller number of steel plates in concrete, thereby dispensing with a lot of steel and fabrication cost.

Further, the particle beams have to be refocused repeatedly to counteract the natural tendency of beams to diverge. More than 800 quadrupole magnets are foreseen for this task. A similar number of higher multipole magnets completes the set of tools which allow machine operators to tune the beams and to overcome instabilities. Using anodized aluminium in the manufacture of quadrupole coils, in place of copper, would save much cost and production effort; detailed investigations into the properties of such coils are already under way.

The costliest item of the machine is the radio-frequency system which will accelerate the electron beams. Its components, klystrons and accelerating cavities, stretch over 1.5 km and their total power consumption at 70 GeV will be over 70 MW. About 19 MW of this will simply be lost in synchrotron radiation, a feature which is negligible in all existing proton accelerators. When a bunch of electrons passes through the cavities, it picks up energy from the applied RF power and thus is accelerated. But the four bunches in each beam spend most of their time outside the accelerating cavities, and the applied power is lost. An ingenious method to reduce these losses is to couple a low-loss mode storage cavity to a set of accelerating cavities. Energy can then be saved by constantly

transferring power between the accelerating and storage cavities.

By itself, this scheme would not help on component cost but it would either reduce running cost, or alternatively allow a higher maximum beam energy. superconducting RF cavities is currently becoming available in a few years' time; they will eventually allow the beam energy to be raised to about 130 GeV. The upper limit is set by the synchrotron radiation power that can be absorbed by the vacuum chamber, and by the field gradient limitations in the accelerating cavities. Research into superconducting RF cavities is currently carried out under a joint CERN-DESY programme, and a first test cavity is to be installed into DORIS by the end of 1979. However, it is unlikely that this technology will be sufficiently mastered for mass production for the first phase of LEP.

Great importance is also attached to the planning of experimental installations and facilities. Experience at DORIS and SPEAR has shown that the cost of experimental detectors and associated electronics and computers amounts to a sizeable fraction of the total machine cost. Furthermore, the average number of charged particles produced in an electron-positron annihilation at these energies is of the order of 20. Very complex detectors are required to track that many particles, and to identify them unambiguously. So a major effort must be made to reduce the cost of such devices.

Konrad Guettler

Fermilab looks to the future

David Dickson visits the Fermi National Laboratory and talks to its new director, Dr Leon Lederman (right)

THE past year has not been an easy one for the Fermi National Laboratory (Fermilab) at Batavia, just outside Chicago. Twelve months ago Fermilab's then director, Dr Robert R. Wilson, was pushing hard for the rapid completion of the next phase in Fermilab's development. This is to be the construction of a second ring of superconducting magnets which, with their increased field, will allow energies of up to 1,000 GeV to be reached, twice existing energies.

Last November, frustrated by indications that the Department of Energy would not provide him with sufficient funds for the rapid construction of the Doubler, Dr Wilson threatened to resign if such funds were not included in Fermilab's budget for the fiscal year 1979. But the department stood firm, providing Wilson with only \$15 million of the \$30 million that he had requested. And in February he resigned

as threatened, citing the department's "indecisive and "subminimal" support for the Doubler project.

The whole affair left the US high energy physics community feeling somewhat uncomfortable. Many of those who had admired Dr Wilson's achievements in establishing Fermilab were uncertain about the strategy that he had chosen to adopt. Dr Wilson also left a legacy at the laboratory where many felt experimental programmes had been unfairly squeezed to allow development work on the Doubler to proceed as rapidly as possible.

The task of putting all the pieces back together again now rests with Fermilab's new director, Dr Leon Lederman, whose appointment was announced in October and who takes over the position full-time next 1 June (although already working several days a week at the laboratory).



of physics at Columbia University and director of the university's Nevis accelerator. He has also been closely involved with Fermilab since its inception; he was a member of the team which selected the site from over a hundred possibilities in the 1960s, and he also led the investigators who discovered the upsilon particle in the summer of 1977.

Widely known not only as a successful experimentalist but also the owner of a quick wit—asked where the laboratory is going, his immediate response is that “It’s going to stay in Illinois—I hope”—Dr Lederman has few illusions about the problems that lie ahead.

At the heart of many of these lies the issue of funding. Tight budgets have squeezed high energy physics as hard as other fields of science. Dr Lederman says that “basic research is being treated rather well by this administration”; but he points out that with three large accelerator projects now under way, the whole US high energy physics programme is “pushing very hard against its ceiling”, and that “if the budget was even 5% higher, it would be an enormous relief”.

High risks

The Energy Doubler will require a ring of 1,000 superconducting magnets, each 7 metres long. Although recognising that, with so little known about such technology, the field was one of high risks, Dr Wilson felt that the design of the magnets could be refined as the first ones were being produced (rather than waiting for a complete design before setting up production facilities).

However the gamble did not quite come off. Technical problems proved to be slightly greater than initially predicated—such as a slight movement in some of the wires of the coils, affecting their superconducting properties—and took longer than anticipated to perfect.

As Dr Lederman puts it: “There were some concepts in economy that were not realised. We had thought that a much larger fraction of the original magnets would be adequate and that we would be able to instal those to make up one sixth of the ring. But not enough of them were of good quality; and that created a problem with funding”.

Dr Lederman places stress on the need to maintain a high level of performance, not only with the Doubler but also with the existing accelerator. “We have at present a responsibility to provide a large fraction of the US high energy research community with 400 GeV protons. I have found attention to the programme has slipped somewhat and people have been travelling here from the East coast and the West coast and waiting for a machine that was broken down a bit too often.”

With development work on the Doubler’s magnets now completed, a team at Fermilab is now preparing a final detailed design of the accelerator, which is expected to be ready in January.

Until a few years ago, many of Fermilab’s problems might have been solved by a quick visit to sympathetic Congressman, with a few million dollars subsequently added to budget appropriations. But this style of approach is no longer appropriate, particularly as the decision making role has shifted from the old Congressional Joint Atomic Energy Committee—which produced much of the political support for early accelerators—to the Department of Energy’s High Energy Physics Advisory Panel (HEPAP).

Earlier this year Dr John Deutch, director of the department’s office of Energy Research, announced that the Office of Management and Budget had accepted a proposal to provide high energy physics with a constant budget over the next few years. Within this limit, the department has left it largely up to HEPAP to advise on how the resources should be allocated; the result is that any major development requires the active backing of HEPAP if it is to get support in the administration.

“One of our current very high-level efforts is therefore to bring the physics community into a level of acceptance, understanding and support of the Doubler project”, says Dr Philip Livdahl, head of the project and currently acting-director of Fermilab.

Dr Lederman has already asked for an outside, independent review of the whole Doubler project, and has invited scientists both at Fermilab and in the universities to present ideas for the type of experiments that they would like to carry out when the 1,000 GeV level become available with the completion of the new ring.

A number of such ideas were discussed at a meeting of HEPAP which was held at Fermilab at the beginning of last week. It is said that the exercise had a beneficial effect on some of those who still have doubts about the Doubler project. The advisory panel has now asked the laboratory to prepare a detailed report on the ways in which the Doubler construction programme might be integrated with other activities, setting out the option at different budget levels so that it can assess the requests for greater support.

Colliding with Japan

From Fermilab’s perspective, HEPAP’s support is likely to be particularly important next year, since it is hoped that funds to begin construction of a colliding beam facility—involving collisions between particles in the existing ring and those circulating in the superconducting ring—can be included in the Department of Energy’s budget request for 1981.

One bright spot on the funding horizon is the proposed collaboration

with Japanese scientists. High energy physics figures prominently in a programme of research collaboration in the energy sciences between the US and Japan that could be worth \$ 1,000 million over the next ten years.

Collaboration in high energy physics will take place at three different levels: carrying out joint experiments on existing facilities, joint research and development in accelerator design and technology, and—potentially the most ambitious point—funding of new facilities.

In the first category, Dr Lederman hopes the Japanese contribution will add those few percentage points to the research budget which would have a magnified effect on scientific productivity. As to the third, Japanese funds could help bring forward some of Fermilab’s more long-term plans: in particular, the development of facilities for the production of high-energy anti-protons which could be injected into the same ring as the protons, but circulating in the opposite direction to give collision energies of 2,000 GeV.

World Machine

And there is an interest in high energy physics not only in Japan, but also in the Republic of China, which has recently sent a number of scientists to study at Fermilab. Dr Lederman has been closely involved in the activities of the International Committee for Future Accelerators, and in particular its scheme for the possible construction of a “world accelerator” in the 20,000 GeV range (20 TeV), well beyond the economic scope of any one country.

“When we started there were only three areas of the world interested in the possibility of such a project: the US, the USSR and Western Europe. With three there are problem of co-operation; but with five the situation is much better, since it does not matter too much if one decides to drop out.”

Dr Lederman is also understandably enthusiastic about the particular possibilities raised by the Japanese connection, and is a member of the Department of Energy working party which has been discussing details of the proposed collaboration between the two countries with Japanese scientists.

He feels that high energy physics could play an important role in Japanese society. “Talking to Japanese politicians and ministers, they seem to feel the lack of intellectual heroes, the role played by Einstein, Fermi, Newton and so on in the West. They do not have that heritage to inspire the young people.” High energy physics, Lederman believes, might provide the inspiration. □

news in brief



Before the launch: checking out the probes that have now surveyed Venus's atmosphere.

Exploration of Venus: Ten spacecraft—six American and four Russian—are exploring Venus this month. One spacecraft, the US Pioneer Venus I, went into orbit on 4 December and is expected to be operational for at least a year. Five other US probes for exploring the Venusian atmosphere penetrated the atmosphere on 9 December and transmitted data during their hour-long descent to the surface. They were not expected to survive the crash landing, though one continued to radio information back to Earth for an hour after reaching the surface. The probes contain instruments to measure density, pressure, wind speeds, heat flow and atmospheric composition.

Meanwhile, towards the end of December, two Soviet spacecraft will "fly-by" Venus, and each is expected to release a lander. These landers are intended to survive for a short period on the surface of the planet and take photographs of their landing site. The instruments on the Soviet probes will need to withstand the extreme conditions on Venus: atmospheric pressures 100 times that on Earth, high concentrations of sulphuric acid in the air and temperatures of nearly 500 °C.

"Anti-intellectualism" hits US basic research: Federally-supported basic research in the US is feeling the edge of a definite anti-intellectualism in some sectors of American public opinion, according to Dr Richard Atkinson, director of the National Science Foundation, in a speech at the University of Indiana. He added, however, that, in supporting those who attacked esoteric-sounding research programmes, the public seemed to be demanding some assurance that Government-supported activities were connected with real human concerns and problems. "The scientific community will be far more likely to gain acceptance by dealing with these concerns in a patient and serious way than by reacting in an ill-tempered or patronising fashion."

Dr Atkinson, whose agency received a budget cut in Congressional committees that reduced the growth of its basic research to below the level of inflation, said that the importance of basic science to the economy was now being recognised in China after the Gang of Four's hostility to scientists and scholars. "The Chinese experience should be a sobering reminder to all of us, scientists and non-scientists alike, that intellectual creativity is every bit as significant as a measure of a society as military strength or economic output," he said. "It seems somewhat paradoxical to me that the present Chinese leadership has grasped this lesson so thoroughly at a time when American opinion seems increasingly uncertain of the value of basic research and scholarship."

European control of genetic manipulation: Guido Brunner, EEC Commissioner for science and technology, has suggested that the Community should formulate rules to cover the experimental and industrial use of genetic manipulation. All such work would have to be notified to the national authorities of the member countries, he said. At the moment, Britain is the only one which requires notification, but others are planning to introduce legislation. A spokesman said the Commission was only at the stage of talking about the proposals, and it is not known if it intends to set up a committee similar to Britain's Genetic Manipulation Advisory Group.

Government calls for cut-backs in animal experiments: Scientists in Britain are being urged by the government to restrict the use of living animals in experiments. In a letter to all scientists in the country licensed to carry out experiments on living animals, the government says the experiments should be "limited to the minimum compatible with the pursuit of legitimate scientific ends". The letter stresses the importance of finding alternative methods of conducting scientific experiments, such as tissue culture or computer modelling. Scientists are asked to take "every reasonable step to confirm, before using living animals, that their investigations cannot be done by any alternative means".

The government hopes scientists will be encouraged to develop new alternative techniques. But, Dr C. Stratmann, scientific consultant to the Fund for Replacement of Animals in Medical Experiments (FRAME), said: "We are concerned that little funds are available specifically to find alternatives." Over the last ten years, FRAME has built up a library of information and literature on alternatives. Dr Stratmann urged scientists to contact FRAME if they wish to find out about alternatives.

The number of experiments on animals has remained fairly constant over the past eight years—at about 5.5 million experiments a year, according to government statistics on animal experiments published last Thursday. Over half of the experiments were carried out for commercial reasons such as drug and cosmetic testing.

Inquiry into extension of Natural History Museum: The government has called for a public inquiry into the Natural History Museum's plans to build a new wing known as the East Infill Block. The plan has aroused public interest because it involves demolishing part of the original Waterhouse building, which is listed as of historical interest. Although an inquiry is not required by law, the government wants everyone concerned to have a chance to put their case.

Pesticides in the diet: The average British diet still contains unacceptably high levels of Dieldrin, a pesticide used against woodworm, according to the latest report of the Government Chemist. Although the level of Dieldrin in the diet is less than half the Acceptable Daily Intake recommended by the World Health Organisation, it has not declined in recent years, despite increased restrictions on its agricultural use. Dieldrin is one of the most poisonous organochlorine pesticides, especially as it is not metabolised by the body.

The persistence of Dieldrin in the diet suggests that it may come from a non-agricultural origin. In an isolated incident earlier this year, 4,000 chickens died in East Anglia after eating litter containing wood chips treated with Dieldrin. Government chemists, therefore, began an inquiry into whether some cattle feeds made from dried poultry litter could be a source of Dieldrin in milk.

How molecular biology won its place in the USSR

PROFESSOR Tumerman was originally a physicist. He later turned his attention to molecular biology—a change which, he explained, reflected the whole story of his life in Russia.

Owing to the stress placed by Soviet defence policy of the early 1950s on the atomic bomb, physicists enjoyed what was, for those days, a relatively privileged position. They had greater freedom of action than other scientists and according to Tumerman, played a leading role in the fight against Lysenko.

In the early 1950s a number of physicists became interested in molecular biology and started working underground. Frequently, said Tumerman, the physicists acted as hosts to such unauthorised gatherings. "Timofeev-Resovskii's first lecture on genetics was given in Kapitza's Institute of Physical Problems", he said. "I myself organised the first seminar on genetics at the Lebedev Physical Institute. We had about 200-300 participants at every meeting of the seminar. This was the first legal seminar on genetics. The time was ripe to begin the fight".

Many famous physicists, including Kurchatov and Tamm, were by now interested in molecular biology, and the pressure was kept up in many forms. An underground seminar was held every year in the Ilmsk nature reserve.

By this time, Professor Tumerman's interests had switched almost completely to molecular biology, although this change was not reflected in his official work. "Before my arrest", he explained casually, referring to his seven years in solitary confinement, "I had worked for 20 years with Sergei Vavilov, as a pure physicist. (Sergei was the brother of Nikolai Vavilov, you remember, who perished during the Lysenko period). We worked together from 1926 to 1947 on fluorescence as a physical phenomenon. The first fluorescent lamps in Russia were made by my hands."

Genetics—learnt in gaol

"In the Vladimir prison, there was a very good library—built up from books confiscated from the homes of prisoners. I began to read books on genetics. There was one very good book by Sunnot and Dent. These books impressed me so much that after I was released I changed my field of interest. I felt that purely physical phenomena were no longer of interest to me. I could only work at the interface of physics and molecular biology. I wanted to understand the role of physics in biological processes. So from 1954 to

PROFESSOR Lev Tumerman, who played a leading role in the fight against Lysenkoism, emigrated from the Soviet Union to Israel in 1972. Since then, he has been prominent in the press discussion of the Kyshtym nuclear "disaster" of the 1950s. Vera Rich talked to him recently at the Weizmann Institute, where he now teaches.

1958, I worked at the Lebedev Physical Institute, but I changed my field of activity to photosynthesis. This was my official work, but unofficially I was interested in the whole problem of genetics and molecular biology."

In the seminars which Tumerman and his fellows organised, little could be done except to present and review the results of foreign papers. Yet, although no experimental work could be done at that time in molecular biology, Tumerman sees the seminars as far from fruitless. "We felt it was very important to involve young people in this field—to give an impetus to press the authorities. And in 1958 we won our victory, and molecular biology was allowed to exist officially."

As an immediate consequence of the new policy, Academician Engelhardt was given the task of setting up a new Institute of Molecular Biology, and Tumerman himself was invited to be head of the Laboratory of Bioenergetics. This laboratory investigated a number of problems, including nucleic acids and photosynthesis; Tumerman's own work was an attempt to apply physical mechanisms to problems of molecular biology.

"During the last few years", Tumerman added, "genetic engineering has evolved. But this came up after I had emigrated and I know nothing about it. I never worked on military applications. It was not easy for me—they often pressed me, but I managed to stay clear of military problems."

Yet although his own work remained unclassified, Tumerman did in fact come into possession of certain data which, whether technically classified or not, the Soviet authorities clearly wanted to keep secret. This was the story of the Kyshtym disaster.

"In the early 1960s", explained Tumerman, "Timofeev-Resovskii was working at the Ilmsk nature reserve on the action of radiation on plants, and there was certainly no contamination there". (This is the nature reserve where the clandestine meetings on molecular biology and genetics had been held.)

At that time, Tumerman's brother was working as a construction engineer

on the new atomic power station at Beloyarsk, some 100 km away. One day, Tumerman set out to visit his brother by car, leaving at midnight. "By 5.30 it was light", he recalled, "and I saw a sign by the road 'No stopping for 25-30 km'".

"On both sides there was *mertvaya zemlya* (dead land). No villages, only chimneys—the houses had been pulled down to keep the people from returning".

Asked to clarify the expression *mertvaya zemlya*, Tumerman explained that by 'dead land' he did not mean there was any signs of devastation. The land was simply empty—no agriculture or any other sign of land-use. "The local people said it was due to a 'catastrophe' at Kyshtym", he said. "I don't have any knowledge of its cause or nature. It had been some years before. I assumed that some waste must have fallen and that the level was so high that the people had had to be evacuated and many hundreds of square kilometres of land left free from cultivation".

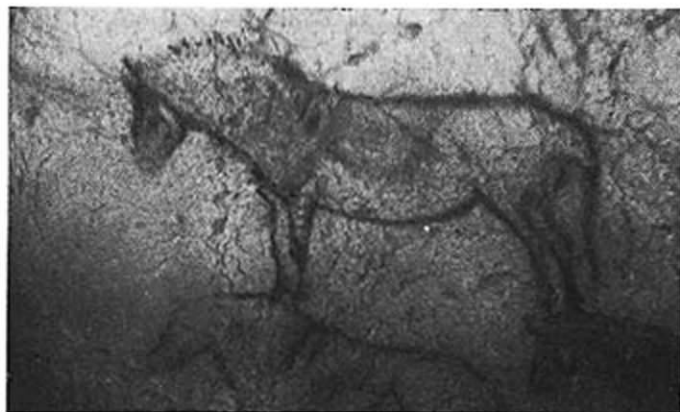
Keeping quiet about Kyshtym

Tumerman at first made no attempt to publicise this knowledge. Only when, some years after Tumerman settled in Israel, Zhores Medvedev began to draw Western public attention to the disaster, did Tumerman begin cautiously to publish what he knew.

"I was afraid," he explained, "that people would use the story to interfere with the building of atomic power stations. I believe that the building of atomic power stations—real power stations, not plutonium processing plants—is the prime necessity for mankind. We must be prepared that in maybe 20 years the oil will run out. And world politics will change. No one will be interested in the Middle East".

"I published my account", he insisted, "to prove that the danger is not in atomic power stations. Atomic power stations are the safest form of energy production, much safer than oil".

However one accepts this estimate, Professor Tumerman's assertion that Kyshtym was not due to some power station disaster seems, on the face of it, irrefutable. For at the time when he passed through the devastated area, he was on his way to visit his brother at Beloyarsk, where the first atomic power station in the Soviet Union was "only at the first stage of building". "So whatever caused the disaster", Tumerman concluded, "it simply could not have been connected with any power station!" □



On cave paintings and nuclear security

By Alvin Weinberg, director of the US Institute for Energy Analysis, Oak Ridge, Tennessee

Dr J. Altuna took well-justified pride as he showed us the cave paintings in the Ekain caves near San Sebastian that he and his colleagues had discovered in 1968. The horses (pictured above) by some Cro-Magnon genius 11,000 years ago were in every respect remarkable: according to Dr Altuna, the best cave paintings of horses to have been discovered to date. The paint we now see is largely manganese dioxide which was probably mixed with animal grease to apply it to the wall.

I was in the Basque country at the invitation of Aranzadi, the Basque Scientific Society. I was to participate in a discussion of nuclear energy with Professor Erik Arrhenius of Sweden. My reaction to the cave paintings, aside from astonishment at the skill of the artist, was therefore coloured by the nuclear debate. Could these artefacts of man, some of which have lasted as long as 11,000 years with little deterioration, bear on the disposal of nuclear wastes? Dr Altuna was very doubtful—after all, not all the paintings are in perfect condition. On the other hand, some were; and in the absence of proof of the unprovable—we cannot know *absolutely* the fate of geologically sequestered wastes 10,000 years from now—we can draw inferences from long-lasting artefacts of man. Herbert Muller, in his *Freedom in the Ancient World*, speaks of the ancient cave-artists as having “unconsciously . . . worked for posterity”. In this instance they have, unwittingly, proved that at least some works of man can survive for immensely long times; 11,000 years is sufficient for the radioactivity in wastes to fall below that of the original uranium ore whose fission gave rise to the wastes. And if some artefacts can last that long without change, is it not more plausible to believe that we, who are more sophisticated than our Cro-Magnon

ancestors, can reproduce the necessary conditions for survival of solidified wastes than that we cannot?

Ekain, or the 30,000-year-old stone Venuses, or, for that matter, the 2,000-million-year-old natural reactors at Oklo in Gabon add to what I believe is already convincing evidence that waste disposal is tractable. The issue ought not to be used as an argument against the use of nuclear power, despite the controversy that once again rages over the feasibility of waste disposal.

Rather, the central argument, and the one that I suspect all the other issues will coalesce around, is the peculiar requirement for institutional stability demanded by nuclear power. It is therefore ironic that just 30 kilometres from Ekain, with its evidence for the technical feasibility of waste disposal, lies Lemoniz, a prime reminder that nuclear energy and social instability don't mix. About a year ago, a main steam generator in one of the pressurised water reactors under construction at Lemoniz was bombed by Basque terrorists; and a terrorist was killed in a shoot-out with guards at the construction site.

The Lemoniz incident was not primarily anti-nuclear: it was rather that Lemoniz (above right) was sited under the Franco regime without much consultation with the local Basques. It had become a symbol of Basque nationalism: in the view of many Basques, Madrid was imposing its will on them.

This essentially political objection to Lemoniz provides fertile ground for anti-nuclear activism. And in the colloquium, Professor Arrhenius and I were bombarded with the same questions I encounter everywhere: waste disposal, reactor safety, proliferation, low-level radiation effects.

The Basques are of two minds about Lemoniz. On the one hand, they see it

as imperialist intrusion. On the other, they sense that nuclear stations, rather more than, say, oil-fired stations, confer a degree of energy autarky. Once the Lemoniz reactors are built, the Basque country will have a large source of electricity entirely within Basque borders. A precondition for this additional degree of energy self-sufficiency, I pointed out to the Basques, is an end to nuclear terrorism. Once Lemoniz is operating and its core contains billions of curies of radioactivity, it must be judged out of bounds for would-be terrorists: a core meltdown induced by terrorist action would harm the Basque country more than anywhere else.

Would the presence of an operating reactor mitigate the scope of action of terrorist groups—at least to the extent of putting nuclear plants out of bounds? I should think this might be the outcome in the Basque country where terrorists and local population presumably have the same aim—more autonomy for the Basques. Once Lemoniz is operating, one would expect terrorist acts against it to cease. I am less sanguine about nuclear terrorism in other situations where the terrorists may have no motive except destruction of the existing order. The only safeguard against such terror is heavy security. It is no accident that Lemoniz is as closely guarded as any US atomic weapon establishment. But these security measures are confined to the reactor site, and to speak of such measures as implying a police state seems to me to be an absurd exaggeration. Heavy security against terrorists is a price that nuclear energy exacts; fortunately, the number of places that will have to be so guarded is small, and they pose little threat to the society as a whole. I would hope that nuclear plants everywhere are made as secure as Lemoniz now is. □

correspondence

Good and bad Nobel prizes

SIR,—The Soviet authorities and mass-media are distinguishing between 'good' and 'bad' Nobel prizes. A Nobel prize is a good one and means great and deserved recognition for the USSR, if it is awarded to an obedient and loyal Soviet intellectual: to the writer Sjolokhov, to the physicists Basov and Prokhorov, to the chemist Semjonov or the economist Kantorovich.

The Nobel prize is a bad one and is a provocation of western imperialists and international reaction, if it is awarded to some of the Soviet writers or scientists, who are on the 'banned' index; say Boris Pasternak, Alexander Solzhenitsyn or Andrei Sakharov. Pjotr Kapitsa's recent Nobel prize for physics is considered good by Soviet authorities and mass-media.

Professor Kapitsa is no doubt a great and famous scientist. He was awarded the Nobel prize for a discovery he made 40 years ago in Moscow with equipment which had been sent to him by his professor, Ernest Rutherford, from Cambridge. But is this year's Nobel prize really a good one for the Soviet Union? Does Pjotr Kapitsa really belong to the obedient and loyal Soviet intellectuals?

The facts of his life suggest the opposite. The young Soviet physicist came to Cambridge in 1921 and in a few years became Rutherford's most respected collaborator and expected successor in the Cavendish Laboratory. In 1934 Kapitsa left for a vacation in the Soviet Union and unexpectedly was prevented by Soviet government from returning to Cambridge. The Soviet government offered him a new institute in Moscow without any financial and administrative restrictions.

Rutherford had been trying very hard to get his collaborator back, but when he finally saw the vanity of his attempts, he finally decided to make a beautiful gesture: he sent the equipment of Kapitsa's recently-built Mond laboratory to Moscow. Kapitsa had just completed the equipment before his departure on vacation.

Kapitsa had his institute in Moscow, an important position in the Soviet academy of sciences, and a considerable influence in the government when, in 1939, another of the future Nobel laureates and his close friend Lev Landau had been arrested. Kapitsa came to the Kremlin and presented an ultimatum: if Landau was not released, Kapitsa would leave his institute and stop his work. This was a risky step, but the Soviet government needed Kapitsa. Landau was released and his life saved.

In spite of all this Kapitsa was himself a privileged prisoner. Until the late fifties he was not allowed to travel abroad, his contacts with foreign scientists were restricted, and his correspondence often confiscated. Kapitsa became a prisoner in his country house at Nikolina Gora in 1945, when he fell out with Stalin over the problem of Soviet nuclear weapons. He was released from prison only after Stalin's death and only then was able to resume his directorship in the Institute of Physical Problems.

Kapitsa never hid his critical attitude to the Soviet reality, nor his sympathy with those who openly oppose this reality.

"How many roubles would our finance ministry release to Rembrandt for the brushes, how much for the canvas and how much for the colours?" says Kapitsa, chiding administrative restrictions confining Soviet science. "On 20 February 1956 an instruction was issued, according to which all directors of academic institutes must present a plan of every single working day for the approval of the academy. But I, for example, cannot plan every single working day, say when I will be sitting in the laboratory and when in my studio—this depends on how our investigations will proceed . . ."

Very sharply, and often, Kapitsa criticised the backwardness of Soviet science, its low productivity. In 1965 the USSR and USA had approximately the same number of scientists, but the USA did about one-third of world science, and USSR only one-sixth. "Therefore, no matter how sad it may sound, we have to admit that the productivity of our scientists is two times lower than that of Americans."

Andrei Sakharov is a good friend of Kapitsa, and so are many other Soviet dissidents. Kapitsa was one of the initiators of the public protest which helped to release Soviet biologist Zhores Medvedev from psychiatric prison. His signature was among the few tens demanding the abolition of the death penalty in the USSR. And his villa in the institute park is a frequent meeting place for dissident Soviet intellectuals. The atmosphere inside is stimulating: in Kapitsa's studio hangs a photograph of Solzhenitsyn, with his handwritten dedication.

When the Soviet sculptor Ernst Neizvestnyj was expelled from the USSR in 1974, Kapitsa gave a farewell party for him in his house. In the presence of many guests he made a toast, in which he said: "I have to admit that I envy you a little bit. You are young and you will live to the time when you could come back to Moscow. And you will walk on the Solzhenitsyn square, and on Andrei Sakharov prospect, and the street where you will have your atelier will bear your name. But I am already old and will hardly live to see it . . ."

Eighty-four years' old academician Kapitsa may really not live long enough to see such changes in USSR. But when they finally come, nobody will deny his merits.

This year's Nobel prize for Pjotr Kapitsa is, therefore, not quite a 'good' prize for the Soviet Union. But is more than a highly deserved prize for an old professor.

Name and address withheld by request

Heracitus Revisited

SIR,—You may be interested in these lines stimulated by the review of Erwin Chargaff's new book (*Nature* 276, 133; 1978):

I'll tell thee everything I can:

There's little to relate.

I met an old, embitter'd man

Bemoaning his sad fate.

"Who are you, aged man?" I said,

"And what is your pet peeve?"

His answer trickled through my head
Like water through a sieve.

He said "I met a fervid pair

Who built a model crude

The younger one had lengthy hair

And both of them were rude.

They claimed that they were out to find

What makes genetics tick

And when I call the scene to mind

The mem'ry makes me sick."

But I was thinking of a plan

To write a grant request

And if the funding once began

I'd count myself well-blest.

So, having no reply to give

To what the old man said

I cried "Come tell me why you grieve"

And thumped him on the head.

He said "The times are bestial

The world has gone to hell.

My culture is celestial

In science I excel.

Of Mozart's symphonies and themes

I know the form and keys.

I dream the most exalted dreams

Of any Viennese."

But I was thinking of a way

To cut him down to size

By noting what he had to say

Was something less than wise.

And then I wondered if these lines

Would capture his attention

Because his musings show some signs

Of scanty comprehension.

And so if e'er by chance I put

My fingers into glue

Or madly squeeze a right-hand foot

Into a left-hand shoe

Or if I drop upon my toe

A very heavy weight

I weep, for it reminds me so

Of that old man I used to know

Who seems distracted with his woe,

Who mumbles of the long-ago

As if his mouth were full of dough

Bemoaning his sad fate.

The Wight Night

THOMAS H. JUKES

University of California,
Berkeley, USA

Homo erectus

SIR,—I was very pleased to see that due recognition was given to *Homo erectus* in the item on East Javan hominids (28 September, page 306).

Few, indeed no, previous commentators have given this hominoidal form sufficient weight, although his more commonly used name gives a clue to his place in taxonomy: just a breath away from Pe-King Man must come Bel-Ching Man.

His undoubted place as a direct ancestor of *H. sapiens sapiens* is attested by *H. erectus*' easily identified descendants in any group of humans today.

However, I have been greatly puzzled over how his patronymic attribute has been deduced from skeletal evidence.

N. R. LONDON

London, UK

news and views

Solar energy storage and conversion by hydrogen cycle

from Robert W. Cahn

ALL forms of 'current' energy—Sun, winds, waves, tides—are intermittent and variable and their use for large-scale and domestic purposes alike demands provision of energy storage. This may well turn out to be more difficult than and just as capital-intensive as the devices for absorbing and converting the energy in the first place. The recent controversy in *Nature* concerning the storage requirements associated with a system of windmills indicates the crucial importance of the storage capacity if steady power is to be generated from variable input.

Most domestic solar heating installations to date are intended largely or wholly for water-heating, and the thermal mass of an insulated hot-water tank provides all needful storage capacity. If, however, space-heating is proposed as well, or even electricity generation, then a more sophisticated storage facility is needed. Heat can be stored in pebble beds, water tanks or salt baths and retrieved by forced heat exchange when required. However, space is expensive and a high storage capacity per unit volume (specific capacity) is therefore at a premium. A high specific capacity implies exploitation of chemical reaction energies as distinct from simple specific heats, and it is in this connection that metal hydrides have received a good deal of attention in the past four years.

A number of metal hydrides (TiFe, LaNi_5 , Mg_2Ni and Mg_2Cu are the most studied) have convenient dissociation pressures and temperatures, substantial heats of dissociation and can store more hydrogen per unit volume than can a liquid-hydrogen Dewar vessel (Hoffman *et al.* *Int. J. Hydrogen Energy* **1**, 133; 1976). A number of laboratories, of which Brookhaven National Laboratory has been the most active, have been examining the metallurgical and engineering features of hydride storage installations, partly with automotive uses in mind. A recent paper from Argonne National Laboratory (Green, Schreiner & Shaft *Int.*

J. Hydrogen Energy, **3**, 303; 1978) presents a conceptual study of a system using such hydrides for storage of heat and its use in adjustable proportions for domestic heating, air-conditioning and electricity generation. It is a welcome publication, since much work in this field is issued in inaccessible reports or expensive conference proceedings — what might loosely be termed scientific *samizdat*. All 12 references at the end of this paper are to unpublished reports, house journals or conference proceedings, and this practice leaves little scope for the serendipitous browsing that so often generates new scientific ideas. For this reason, the existence of regular journals like the *International Journal of Hydrogen Energy* is particularly welcome; in a field like this, the absence of a highly specialised journal is apt to be associated with entrenched scientific *samizdat*.

The Argonne apparatus, in the simplest of its several forms, involves a solar collector with concentrator that can warm a heat-transfer fluid to 140 °C. The warm fluid then heats up a primary LaNi_5H_6 bed, releasing hydrogen at about 50 atm. The released hydrogen is cooled to 50 °C and expanded to a pressure of 40 atm or less. The cool, lower-pressure hydrogen is then converted to hydride in a secondary bed (a cheaper bed using mischmetal, a mixture of rare earth metals, instead of lanthanum, is proposed). The heat released during expansion and hydration is absorbed in a separate loop of heat-transfer fluid, and this heat (at 50 °C) can be used for space-heating. Hydrogen is released from the cooled MmNi_5H_6 secondary bed by lowering the pressure to about 8 atm and can be recombined with the primary LaNi_5 bed at 50 °C at the same pressure. That bed is then reheated by solar energy and the cycle recommences.

This represents a simple non-storage cycle. In fact, the storage bed would be kept hydrogenated till nightfall and the apparatus would then be operated in heat-pump mode; the primary bed is kept at 50 °C by manipulating heat-

transfer fluid and is used as a source of space heat, while the secondary bed can progressively dehydrogenate at low pressure and 0 °C; this low temperature is achieved by absorbing heat from the environment.

To achieve continuity in operation (as would be required for continuous air-cooling or for electricity generation) at least two primary and two secondary beds would be used, with appropriate switching of hydrogen and heat-transfer fluid flows so that each undergoes a staggered cycle: absorption, heating, desorption, cooling. The cycle length is presumably limited by maximum heat-transfer rates at one extreme and by low demand for domestic heat at the other extreme. A variant of this cycle allows the apparatus to be used for air-conditioning instead of space-heating.

A more complex system involving three beds of each kind would permit a proportion of the absorbed solar energy to be used for electricity generation. (Warm hydrogen would be passed through a turbogenerator: the alternative of oxidising the hydrogen to water in a fuel-cell would be inappropriate in a closed cycle system). The authors undertake a detailed assessment of realistic efficiencies to be expected, allowing for heat losses involved in heating and cooling the storage beds: the maximum theoretical efficiency is 28% (electric power as a fraction of solar heat absorbed by the primary bed), the expected realistic efficiency is 16.5%. If the apparatus is used simply as an air-conditioning (cooling) device, the heat-pump mode ensures that, at ambient temperature, for each mole of hydrogen about 3.3 times the dissociation energy can be extracted as useful cooling power.

Nothing is said about economics. The high cost of mischmetal and even higher cost of pure lanthanum would seem to place a question-mark against the particular hydrides chosen, in spite of their particularly convenient dissociation temperatures.

The Argonne design prompts a number of reflections. The apparatus would be of very considerable com-

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plexity. Each bed would be associated with several heat-transfer loops, pumps and pressure valves, and there would need to be an elaborate automatic control installation to switch both hydrogen and heat-transfer fluid into appropriate circuits at appropriate times, to secure the staggered cycles and also in response to changes of incoming solar power level, space-heating or cooling demand, hydride temperature, hydrogen pressure and fluid temperature in various parts of the apparatus. This is quite apart from the control provisions needed to keep electric output constant in the face of varying hydrogen temperatures and pressures. There is a widespread illusion that renewable energy sources such as Sun and wind are necessarily associated with primitive or intermediate levels of technology: people are apt to think in terms of solar cookers for use in Indian villages or of water-heaters for swimming-pools or for Israeli dwellings. In fact, if solar power is ever to be more than a negligible fringe energy source, plainly it must be accompanied by very advanced technology. This is true both of the initial capital input (such as the manu-

facture of silicon solar cells) and of the maintenance of complex installations, which will place high demands on initial design (perhaps in the form of replaceable modules), on fault diagnosis and on high reliability in manufacture. Such things will certainly be 'mills' in William Blake's sense, though bright and beneficial rather than dark and satanic.

In the very early days of domestic electrical power, a century ago, there was a brief sharp argument as to whether separate electric generators were to be installed in the basement of each home, or central generation and distribution was preferable. One may wonder whether, before another century is out, this argument will be reopened in sunny parts of the world. Meanwhile, the most promising application, in the short term, of installations like the Argonne design would be for air-conditioning in hot, isolated and fuel-deficient places like North-western Australia. Such an air-conditioning facility, built into dwellings and communal spaces, may well be the factor that determines whether or not labour can be attracted to work in mines in such inhospitable places. □

nucleation is about 90 kcal mol⁻¹. During the latent stage the supersaturated solution of haemoglobin monomers (that is, tetramers, molecular weight 64,500) is unstable with respect to the phase of isotropically oriented fibres. The latter, in turn, is unstable with respect to the formation of the anisotropic state, as evidenced by the slow development of birefringence.

Minton⁵ has applied fundamental models for the entropically driven formation of tactoids from long rod-like molecules or particles to the haemoglobin S system to develop an equilibrium model of gelation. The non-ideality which arises from the large excluded volumes of the highly asymmetric polymers results in a phase diagram containing regions of purely monomeric haemoglobin and of isotropically and anisotropically oriented fibres, the last either in the tactoidal state or in a very highly aligned and concentrated quasi-crystalline condition. The diagram also contains a stem-shaped heterogeneous region reflecting a separation into conjugate isotropic and anisotropic phases.

Pumphrey and Steinhardt⁶ have now discovered and characterised a new state of haemoglobin S which defines a new metastability as well. If concentrated solutions of deoxyhaemoglobin S are stirred above a low threshold rate, birefringent, uniaxial, needle-like crystals appear instead of the familiar gel. Since seeding of a gel with crystals results in conversion to the crystalline state, the crystal form is more stable thermodynamically, although only marginally so, since the well-defined solubility of the crystals in very near the minimal gelling concentration. Our observations⁷ on dilute gels of deoxyhaemoglobin S, observed in 1.7 M phosphate, show a similar metastability: As a function of rates of warming and shaking, there is reversible formation of either a gel or a fluid but more turbid suspension.

In addition, polymorphic forms of the fibre exist (see *News and Views*, *op. cit.*), so that structural as well as physical chemical studies demonstrate

Sickle cell: a metastable disease

from Robin Briehl

SICKLE-cell anaemia was the first 'molecular disease' to be discovered; the term was coined originally for it by Linus Pauling to celebrate the discovery that the condition was linked to the presence of a mutant haemoglobin in the red cells of the patients. In the quarter century that has passed efforts have been made, with less than conclusive results, to understand in detail just how the deviant solubility properties of the abnormal haemoglobin bring about the sickling phenomenon, and what, in the light of such a biochemical or biophysical understanding, one could hope to do about it. I want to consider here one aspect of this multifaceted problem.

In the classical picture the pathogenesis of sickle cell disease depends on deoxygenation-dependent polymerisation of the abnormal haemoglobin S into long fibres and the formation from these of anisotropic, spindle-shaped, liquid crystalline bodies, designated tactoids. The final result is a viscous 'gel' which deforms the erythrocyte and makes it rigid. Gelation and its inhibition have been studied intensively in the past 5 years by attempts to develop covalent or non-covalent inhibitors of gelation, by elucidation of the structure (see *News and Views* 272,

476; 1978) of the intermolecular interactions in the fibre, and, the subject of this note, by studies of the kinetics and equilibria of gel formation.

Under the currently accepted model for the kinetics of gelation, a long stage of highly cooperative nucleation is followed by rapid fibre growth and then by a slow alignment of fibres to the anisotropic state¹. The termination of the nucleation or latent stage, originally observed through an increase in viscosity² is also manifested in heat absorption, the onset of birefringence¹ turbidity increase³ and broadening of water proton magnetic resonance line-widths⁴. The activation energy for

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the existence of different condensed states.

The most common assay of gelation, minimal gelling concentration, depends in part on kinetic factors. A better measure of thermodynamic properties is obtained from the recently developed C_{sat} assay in which a preformed gel is sedimented and the concentration of the supernatant, consisting of monomers previously in equilibrium with the condensate, is obtained. C_{sat} is independent of total haemoglobin concentration and is thus a saturating concentration which reflects a phase separation⁸⁻¹¹. Magdoff-Fairchild *et al.* suggest the possibility of two different condensed phases on the basis of their observation of a sharp change in the apparent van't Hoff enthalpy of gelation at 22 °C. Our studies show that C_{sat} is always markedly greater than the minimal monomer concentration needed to produce measurable aggregation, C_{agg} , and therefore the overall solution to gel equilibrium cannot be treated as a single sharp polymerisation and phase condensation. C_{agg} reflects polymerisation free energy, whereas C_{sat} , although also a monomer concentration, reflects the separation into isotropic and tactoidal phases.

The endothermic enthalpy for the entropy driven process of gelation has been measured by Ross *et al.*¹² from the temperature dependences of C_{sat} and birefringence observations, and by direct calorimetry. ΔH falls from +4,000 cal mol⁻¹ at 15 °C to 2,200 at 25 °C to zero at 37 °C. The large associated ΔC_P is -197 cal degree mol⁻¹. Most of the enthalpy is associated with the polymerisation process, ΔH for alignment being in the neighbourhood of zero. These results are consistent with a polymerisation process dominated by hydrophobic interactions and alignment dependent on entropic forces as modelled by Minton⁵. The results of Magdoff-Fairchild *et al.*⁸ show similar ΔH values and also a pH dependence of ΔH .

The three important physiological ligands of haemoglobin, oxygen, proton and 2,3 diphosphoglycerate (DPG) affect gelation. Thermodynamic studies (of C_{sat} and C_{agg}) show that protons above pH 6.8 favour gelation and that an optimum for gelation exists near pH 6.8^{10,11,13}. Protons also shorten the latent stage delay time¹⁴. In our studies DPG, and more so inositol hexaphosphate, also favour gel formation. On the other hand, under a single set of conditions resembling those in the erythrocyte, Swerdlow *et al.*¹⁵ show no effect of DPG on gelation. Kinetic studies show that DPG shortens the delay time¹⁶. The oxygen

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Ice-Age palaeo-oceanography of the Mediterranean

from N. J. Shackleton

THE CLIMAP project had as its first major goal the reconstruction of global climate at the peak of the last glaciation about 18,000 years ago. One purpose of this was pure geological excitement, another was to use the resulting reconstruction as a boundary condition for numerical general circulation models of the atmosphere (for example, Gates *Science* **191**, 1138; 1976). One of the most exciting moments in the CLIMAP project was the day we first compared the climate simulated by Gates' model for the ice age world, based on oceanographic data, with independent geological observations from the continents.

On the coarse grid-scale with which CLIMAP was forced to work the Mediterranean Sea looked rather insignificant, particularly considering the vast areas of Pacific we had to deal with, and it was at first treated rather summarily. However, it is an area of special interest to other workers, and Thiede has now presented a CLIMAP reconstruction of the Mediterranean alone (page 680 of this issue of *Nature*). The reconstruction is based on the Foraminifera, and its strength lies in the fact that it was possible to obtain a temperature calibration of the planktonic foraminiferal faunas of the Mediterranean utilising sediment samples from the North Atlantic and particularly from the Bay of Biscay. This is important because the glacial faunas clearly include some that do

not lie in the present range within the Mediterranean.

Glacial winter sea surface temperatures ranged from below 7 °C on the entire Spanish and French coasts to more than 19 °C on the Egyptian coast, while summer temperatures were below 13 °C along the Spanish and French coasts, rising to over 25 °C in the South-East. Thus the Western Mediterranean appears to have been very considerably cooler than it is today, although in the South-Eastern corner conditions were not greatly different from today.

This reconstruction will be received with great interest by archaeologists who are exploring man's existence around the Mediterranean during the last glaciation. There is not nearly as much information as one might like preserved in some of the caves occupied around the Mediterranean for reconstructing the past environment, and a reconstruction of sea surface temperatures should provide a welcome input. One hopes that a synthesis will emerge which utilises the marine and terrestrial evidence together; at the least, it should be possible to calibrate Thiede's estimate of the seasonal temperature range by making oxygen isotopic measurements in some of the mollusc shells which prehistoric man so thoughtfully collected for our use.

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equilibria of dilute haemolysates of haemoglobins A and S have long been known to be indistinguishable even though haemoglobin S cells have a lower oxygen affinity than normal cells. Gill *et al.*¹⁷ have now examined concentrated haemolysates and found that a concentration-dependent lowering of affinity exists for haemoglobin S at low saturations, associated with evidence of polymerisation by light scattering, but not at high saturations. The effect depends on the free energy change associated with polymerisation of the deoxygenated form.

In an extension of previous thermodynamic analyses, Minton¹⁸ and Ross and Minton¹⁹ have shown that the non-ideality arising from the excluded volume of monomeric haemoglobin in solution makes a very large contribution to thermodynamic activity at the high haemoglobin concentrations

required for gelation. The non-ideality as obtained from osmotic pressure data and the sedimentation equilibrium results of Williams²⁰ and Briehl and Ewert¹³ can be well accounted for under a model in which the haemoglobin molecule in solution is treated simply as a hard, impenetrable, quasi-spherical particle. Ross *et al.* demonstrate that this non-ideality suffices to explain the effects of some non-S haemoglobins on gelation in mixtures with haemoglobin S; no direct interactions of copolymerisation are required. The non-ideality thus explains the previously observed nonspecific facilitation of gelation of haemoglobin S by the addition of haemoglobin A or F or ovalbumin^{21,22} and receives further confirmation from the demonstration that isolated α and β subunits have the same effect on gelation of haemoglobins S as haemoglobin A²³.

Behe and Englander²⁴ have extended this approach by examining the effects of various non-haemoglobin S proteins on the kinetics of gelation. They show that the delay time in the nucleation stage, formally dependent on approximately the 30th power of haemoglobin S concentration²⁵, is about 10th order in activity. They conclude that the size of the critical nucleus, previously considered equal to the formal order of the reaction, is composed of between 10 and 18 monomers.

Pathogenesis

Eaton *et al.*²⁶ and Sunshine *et al.*²⁷ have already proposed convincingly that the delay time in the nucleation stage, dependent on a high power of the supersaturation ratio (defined as the ratio of total haemoglobin concentration to C_{sat}), can be a major factor in the clinical development or inhibition of sickle cell crises. Presented with metastable behaviour at a number of stages, at least three phases, isotropic, tactoidal and crystalline, containing highly polymerised or condensed haemoglobin, and evidence of microscopic fibre polymorphism, we may now ask whether kinetic factors at other stages of gelation might not govern pathogenesis by facilitating formation of one or another phase and/or by bypassing the most pathogenic phase. Given this polymorphism, we also need to reconsider the classical view that the sickled erythrocyte is a membrane covered tactoid. Such an approach is implied in Minton's analysis of the phase structure of the gel and suggested also by Hermans²⁸ observation that the formation of tactoids in rod-like poly- γ -benzyl-L-glutamate is accompanied by a sharp decrease in viscosity. Harris and Bensusan have observed that haemoglobin S viscosity, reaching a maximum after the nucleation stage, later falls to a lower level. Since this fall apparently occurs in the period of increasing birefringence, tactoid formation here may also be associated with diminished viscosity.

Under this approach, sickle cell disease can be viewed as a disease encompassing multiple phases and phase changes. Clinical variability among different patients and the episodic nature of the disease may depend on kinetic paths and on marginal changes in free energy. Accordingly, control of these factors in the natural history of the disease may be at least as fruitful an approach to specific treatment as the development of a covalent or even non-covalent inhibitor of gelation. In fact, as pointed out by Behe and Englander, modified haemoglobin molecules which do not copolymerise still exert a highly deleterious effect due to the large excluded volume effect.

If these many major advances permit this first described molecular disease to be seen as a disease of physical chemistry, then this view can be considered to have its origin in the work of Allison²⁹ which anticipated many of the modern developments. $\square$

Laser annealing of semiconductors

from D. Weaire and J. I. B. Wilson

In recent years the laser has become the magic wand of the physicist and chemist, facilitating spectroscopic analysis, inducing chemical reactions, separating isotopes. Laser annealing of semiconductors is emerging as yet another exciting new application.

Annealing is the healing process by which a crystalline semiconductor, imperfectly grown or damaged in the course of introducing dopant atoms, is restored to a state of comparative crystalline perfection. This is usually achieved by holding the sample at 800–1,000 °C for 30–60 min (thermal annealing). It now seems that illumination with a laser beam can in many cases produce better results in one millionth of a second!

Credit for this discovery is generally attributed to various Russian groups who published a series of papers on laser annealing in 1975–77 (see for example, Khaibullin *et al.* *Sov. Phys. Semicond.* **11**, 190; 1977). In due course interest was aroused in American laboratories and there has been an avalanche of *Applied Physics Letters* on the subject during the present year.

For example, a group at Oak Ridge National Laboratory has studied the annealing of surface layers of silicon implanted with 35 KeV B⁺ ions. Other American groups have examined the annealing of As⁺ and Sb⁺ ion implants of higher energy (100 and 150 keV) in Si, and implanted GaAs. Ion implantation is a particularly disruptive doping process in which ions are accelerated to energies sufficient to displace the host atoms from their regular lattice positions. By this means shallow, heavily doped layers are produced, but many dopant ions are left on undesirable inactive sites and a great deal of structural damage is produced. Thermal annealing repairs much of this but only laser annealing is effective in removing dislocations and stacking faults. In addition, laser annealing is more efficient in rendering implanted ions electrically active, even to levels above the normal equilibrium solid solubility

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limit of the dopant in the semiconductor.

Shallow p-n junctions produced by ion implantation are of particular interest for solar cells. In this case the damage due to ion implantation is responsible for a shorter minority carrier lifetime in the surface layer and hence lower efficiency, when compared with conventional diffused p-n junction cells.

Laser annealing can produce cells at least equal to conventional cells in performance, with a significant overall saving in the energy consumed during production.

It has also been shown that good ohmic contacts, difficult to produce on GaAs without decomposing the compound, can be made on GaAs wafers and epitaxial layers by laser alloying of deposited metal coatings.

Work in the USA at Bell Laboratories and Western Electric, and in Italy at the universities of Rome and Catania has demonstrated the potential of laser annealing for the recrystallisation of both polycrystalline and amorphous silicon. Amorphous Si films produced by evaporation and by implanting Si⁺ ions have been recrystallised by single 50 ns ruby laser pulses. Polycrystalline Si has a recrystallisation threshold of $\sim 70 \text{ MW cm}^{-2}$, whereas amorphous silicon requires only 45 MW cm^{-2} . This is probably due to the difference in their absorption coefficients.

Any one of a variety of lasers can be used, provided that the semiconductor absorption coefficient is sufficiently high at the laser wavelength. Annealing or recrystallisation is generally limited to a $0.5 \mu\text{m}$ thick surface layer, and this means that there is no undesirable heating of lower layers which might cause unwanted impurity diffusion. Only the highest laser power levels may alter ion implanted dopant profiles, damage the surface of the semiconductor, or produce thermal stress cracks.

Such intense interest at the applied level should surely spawn more basic studies, but this does not yet seem to have happened. For example, at the recent 14th International Conference on the Physics of Semiconductors*, there was little mention of the topic, despite 'Laser Interactions with Semiconductors' as one of the themes. One obvious but difficult fundamental question which is raised by laser annealing is the following. Is it merely a thermal effect, that is, are its beneficial effects entirely attributable to brief localised heating, perhaps including melting? Alternatively, is charge carrier excitation by the ionising radiation a significant factor? Since somewhat similar results have been achieved with elec-

*Held in Edinburgh in September, 1978.

tron beams, and since the extremely high dopant diffusivities observed are compatible with the formation of a molten region, the thermal hypothesis is strongly supported by some experiments. On the other hand, X rays have been observed to enhance the diffusion of metal contacts into the surface of semiconductors and photoionisation may enable charged interstitial impurities to move more easily to vacancies in the crystal lattice. Certainly melting is not essential for removal of ion implantation damage.

This kind of debate has already been conducted in a closely related area. The use of a laser beam to recrystallise locally a chalcogenide glass (a multicomponent amorphous semiconductor such as sputtered Ge-Te-As) was proposed several years ago as an optical storage technique. Even in this case, the relative role of thermal and photostructural effects remains obscure. □

Damn the dogma

from John C. Allen

A TOURIST hotel set in the mountains 350 km North of Oslo was the venue for a recent meeting on Chalone*, a most controversial subject in cell biology. The original hypothesis, which, alas, has been elevated to the status of a dogma, proposed that tissue homeostasis is maintained by mitotic inhibitors termed chalones, which are non-cytotoxic, tissue specific and species nonspecific, and which are secreted by the tissue over which they exert their effect. The theory has been beset by problems ever since its formulation by Bullough and Laurence in 1960, not the least being that in 18 years of intensive research there has been no confirmed report of the isolation of a pure chalone, much less any firm molecular identification. Nevertheless, the scientific importance and potential clinical uses of such substances are manifold, and the literature abounds with reports of partially purified epidermal, granulocytic and lymphocytic chalones. Certainly, the concept has stimulated an immense amount of research, and it was fitting that the participants at the meeting demonstrated their debt to William Bullough (Birkbeck College, London) by presenting him with a portrait to mark his forthcoming retirement.

The organisers had invited two eminent 'devil's advocates' from more respectable realms. A. B. Pardee (Sidney Farber Cancer Institute, Boston) and

R. W. Holley (Salk Institute, San Diego) fulfilled this difficult role admirably and with unanimity. In their summing up, Pardee believed that there was no evidence for the existence of chalones as defined by Bullough, and Holley exhorted all to stop concerning themselves with restrictive definitions with the memorable phrase 'damn the dogma'. However, both felt that endogenous inhibitors do exist and that workers should now set about purifying them from associated nonspecific activity.

Indeed there was no shortage of inhibitors: over 30 were described, with molecular weights ranging from 100,000 to 300! Doubtless, several will prove to be identical, and many trivial. Among the more interesting is that painstakingly isolated from blood by W. R. Paukovits (Cancer Research Institute, University of Vienna.) This is an SH-containing pentapeptide of molecular weight 590 which apparently contains a pyroglutamyl N-terminus. It exerts a fine control over granulocyte proliferation but is inert towards thymic lymphocytes. The nearest we have to a pure lymphocyte chalone is the thymus-derived peptide of J. C. Houck (Virginia Mason Research Center, Seattle). This substance, of molecular weight 1400 and purified by final cellulose chromatography, inhibits lymphoblast formation by 50% in mixed lymphocyte culture at only 1 $\mu\text{g ml}^{-1}$. Houck informally revealed that sequencing is well under way. It can only be hoped that his characteristic optimism will this time be justified, and that a homogeneous preparation is in the offing.

Problems associated with isolation received thorough discussion. G. Isaksen-Forsen (University of Oslo) and A. Quirk (University of Newcastle) independently reported aggregation with concomitant loss of activity during purification of epidermal inhibitors. Other explanations for chalones' elusiveness were suggested, including their secretion as zymogens and their tendency to adhere to larger molecules. However, there is little firm evidence, and such effects may turn out to be 'laboratory specific'.

Unfortunately, much work reported was on impure and even crude tissue extracts. Surely, the dangers of spurious effects from artefacts should be appreciated by now? J. C. Allen (North E. Wales Institute) described work with synchronised lymphoma cells which showed how the simple molecule spermine could exert a very specific inhibitory effect.

H. R. Maurer (Freie Universität, Berlin) warned of the dangers of reliance on the measurement of tritiated thymidine as sole indicator of cell proliferation, and advocated colony counting in preference. It was agreed that no single assay is satisfactory; perhaps the best plan is to adopt a simple *in vitro* screening procedure and to substantiate the results with a series of assays which must include an *in vivo* test.

Recent studies on epidermal inhibitors have provided some intriguing pointers to their true specificity and perhaps to *in vivo* function. A. Thornley (Witwatersrand University) has discovered that his epidermal chalone has an inhibitory effect not only on epidermis, but also on other specifically epithelial tissues including lung, trachea and bladder. This substance may thus be cell line, rather than tissue specific. In addition, S. Bertsch (German Cancer Research Centre, Heidelberg) has partially purified an inhibitor from a keratinised epithelial tumour of the bladder. This was active only on keratinised, and hence differentiated epithelial cells. This is evidence, albeit insubstantial, that the inhibitor was acting to maintain differentiation rather than as a cell cycle modulator.

Thus many workers have already subconsciously rejected the 'dogma' and have adopted a simple operational definition of the useful word 'chalone'. Perhaps the most encouraging aspect of the meeting was the healthy self-criticism. There is now only one reason why chalone research should not move into the mainstream of cell biology, and that is the lack of a homogeneous preparation. This sense of frustration is understandable, but the cell biologists will have to be patient and give the biochemists time to catch up. □

Status of biocontrol in medical entomology

OVER the past two decades, there have been major and continuing advances towards practical biological control of arthropod vectors of disease. Until the late 1950s, biocontrol in medical entomology meant little more than the (sometimes ecologically irresponsible) use of larvivorous fish—primarily *Gambusia affinis* subsp. Since then, though, the growing number of investigators in this field have increasingly concentrated upon the feasibility of control by mass-produced micro-organisms and parasites.

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*An International Symposium on Chalones in Normal and Malignant Cell Populations was held at Geilo, Norway on 29 October–2 November. It was sponsored by the Norwegian Cancer Society and was convened by Professor O. H. Iverson (Institute of Pathology, University of Oslo).

The current status of biocontrol of vectors is nowhere better exemplified than by its place in the World Health Organization's Special Programme for Research and Training in Tropical Diseases (TDR). This aspect of the Special Programme is emphasising innovative approaches to the control of the major vector-borne diseases of developing countries. Little more than a century ago, most of these diseases were still rife in today's industrialised countries, in some of which certain such causes of widespread morbidity and mortality (malaria in the southern United States, for example) persisted in significant foci until after World War II. During 1978, some 20 research contracts were granted under TDR's biocontrol component. About 30 will be implemented during 1979, notably in West Africa.

Hitherto, vectors of malaria, the filariases and trypanosomiasis, and arboviral diseases (likewise the snail hosts of schistosomiasis) have been attacked almost exclusively by the application of toxic chemicals. These, particularly the persistent synthetic organics, have lost popularity for various reasons including political ones. Undeniably, when over-applied, they can have damaging effects on health and environment. Moreover, genetically based resistance to them seems inevitable. Therefore, the prospects for practical biocontrol of vectors (within the broader concept of ecologically soundly based integrated control) have secured wide interest.

Applied invertebrate pathology is the aspect of biocontrol of vectors that has developed most strikingly in the past 20 years. In consequence, certain bacteria, fungi, protozoa and nematodes can now be seriously considered for widespread use against medically important arthropods. The status of this field was discussed at a recent meeting*. Two groups of micro-organisms already showing promise in agricultural and forestry entomology were not dealt with—entomopathogenic viruses because not yet ready for commercialisation in public health entomology, and protozoa because the results of a 1977 Round Table on their potential for biocontrol are about to be published in a post-congress volume from the 5th International Congress of Protozoology (New York). Relevant examples from the other major taxa follow.

Possible agents

Laboratory and field testing of the efficacy of *Bacillus thuringiensis* var. *israelensis* and *B. sphaericus* (notably strain 1593) has demonstrated high pathogenicity to larval mosquitoes, including in the case of the latter species, anophelines in Nicaragua.

WHO/TDR supported the work there, and is arranging for designation of a strain of *B. t. israelensis* which will shortly be tested against *Simulium damnosum* in West Africa following very encouraging tests of this bacterium against larval blackflies in Newfoundland. Both bacteria are mass-producible by available technology. So far, neither has been shown to pose health or environmental hazards, questions to be further investigated as standardised powder preparations are considered for commercial production.

Some of the fungi eligible for evaluation for vector control (for example, *Culicinomyces* of mosquitoes, *Simulomyces* of blackflies and *Pseudocoelomyces* of tabanids) were only recently discovered. This suggests that greater attention to this topic will inevitably reveal yet unknown fungal (and of course other) candidates. Those already under consideration range from specialised aquatics (including mosquito pathogens of the genera *Coelomomyces*, *Lagenidium* and *Leptolegnia*) through Entomophthorales (including *Entomophthora culicis* and two organisms lately described from the USSR, *Pseudocoelomyces milko* and *Simulomyces lairdi*), to Fungi Imperfecti. A representative of the latter, *Metarhizium anisopliae*, has already been tested with promising results against mosquitoes, including anophelines, in Nigeria. Similarly promising tests have been undertaken with *Culicinomyces* against larval mosquitoes in Australia.

Trials have also been carried out from the tropical South Pacific to North America and the USSR, with species of *Coelomomyces* (none of which can yet be mass-produced except *in vivo*, using laboratory colonies of mosquitoes), *Lagenidium* and *Leptolegnia*. Certain of the fungi under assessment, notably *M. anisopliae* (long since cultured in large quantities and widely applied in the USSR), can also be mass-produced on simple media. This is of obvious interest to nations with restricted access to hard currencies.

Various nematode worms, primarily Mermithidae, have been identified and studied from all major vector groups. To date, however, only one species—the warm-water mosquito mermithid, *Romanomermis culicivorax*—is approaching commercial production (the first registration based on it having

been issued in the USA in 1976). Mermithids can be applied in the field by equipment developed for chemical pesticides, from pressurised hand-sprayers to helicopter booms. Major field tests so far undertaken have involved the use of as much as 750 kg of cultures against *Anopheles albimanus* in a land-locked lake in El Salvador, with up to 94% reduction of the host. Smaller-scale tests in progress under WHO sponsorship have demonstrated the feasibility of practical applications of *R. culicivorax* against container-breeding aedine filariasis and dengue-vectors in the Republic of Western Samoa and the Tokelau Islands, South Pacific.

Commercial prospects

Turning now to imminent commercial prospects, there are two main industrial interests. The first is in spore-forming bacteria, exemplified for present purposes by *Bacillus thuringiensis* var. *israelensis*, and *B. sphaericus*. Further moves in this connection will of course be much influenced by the history of lengthy investment and successful commercial production of 'B.t.' products against agricultural and forestry pests. The second interest concerns *Romanomermis culicivorax*, in which two American companies have already shown interest.

One of these firms has stopped all research and product development work on a mermithid product for financial reasons. The second, Nutrilite Products, Inc. (NPI) is commencing its second year of work aimed at commercial development and sales.

Two major problems that still demand a solution are the design of a shipping container that will protect the mermithid eggs during storage and shipment, and elimination of fungus contamination of the cultures. A 2 year \$30,000 WHO/TDR grant has just been awarded to NPI to continue work along these lines. Even if these problems are solved there is some risk that demand for this biological may not be great enough to sustain a profitable business venture. Additional market research will be needed in this area.

In sum—granted necessary encouragement, biocontrol procedures for use against arthropods of medical and veterinary importance could become a significant element in integrated methodologies for vector control in the very near future. However, they must become so, for obvious reasons of environmental unacceptability of, and inevitable resistance to the synthetic organic pesticides. The only real problem to be faced, therefore, is that of adequate support—at a major level, and now. □

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*The XI Annual Meeting of the Society for Invertebrate Pathology and International Colloquium on Invertebrate Pathology was held in Prague on 11–17 September, 1978.

Clutch size in plants

from Robert M. May

THE idea that animals will tend to have smaller litters or clutch sizes in the tropics than in temperate regions has been around for some time. The underlying thinking is that environments tend to be more predictable and biological interactions to be relatively more important in the tropics (so that an evolutionary premium is placed on investing in the care of young), whereas temperate environments are relatively less predictable (so that the evolutionary pressures are to buy as many tickets as possible in what is necessarily a chancy lottery). These notions are developed more fully in, for example *Nature* 257, 737; 1975; 267, 394; 1977.

Much data on clutch and litter sizes of birds and mammals supports this view. Lack put together information on birds which (as summarised by Southwood *et al.* *Am. Nat.* 108, 791; 1974) shows 82 species that inhabit tropical forests to have an average clutch size of 2.3, 260 species from tropical savannas to have an average of 2.7, 21 species from tropical arid areas to have 3.9—an overall average clutch size of 2.7 for 363 tropical species; in contrast, 88 species from middle Europe have clutch sizes averaging 5.6. In a related but more detailed study, Cody (*Evolution* 20, 174; 1966) took more than 200 species of birds in the families Tyrannidae, Icteridae, Anatidae, Thraupidae-plus-Parulidae and Trogloditidae, and graphed the linear regression of clutch size against latitude for each family. The regression lines all have a roughly similar slope, corresponding to the mean clutch size increasing from around two to around five or six as one goes from the equator to 50° north or south latitude. A similar regression analysis of litter size as a function of latitude for North American mammals gives more varied results (Lord *Am. Midl. Nat.* 64, 488; 1960). This work is most easily summarised by christening the slope of the regression line to be s ; s then measures the increase in mean clutch size per 1° increase in latitude (Cody's results correspond to s around 0.06, with a range from 0.03 to 0.09). Lord finds statistically significant patterns of increase in litter size with latitude for 21 species of rabbits ($s=0.25$), 16 species of tree squirrels ($s=0.05$), 18 species of meadow voles ($s=0.13$), 7 species of chipmunks ($s=0.05$), 14 species of deer

mice ($s=0.16$) and 12 species of shrew ($s=0.12$). Ground squirrels, pocket gophers and rats show increases that are not statistically significant, while foxes, cats, and mustelids show no apparent correlation between litter size and latitude. Tinkle, Wilbur and Tilley (*Evolution* 25, 55; 1970) have compiled data for lizards, which show 28 tropical species having an average clutch size of 5.5 while 57 temperate species have 6.1; although in the expected direction, this difference is not statistically significant.

The broad evolutionary arguments that underline these studies of animal reproduction apply equally to plants, and it is interesting to ask whether given groups of plants manifest sys-

tematic trends in 'clutch size' as a function of latitude. Levin and Turner have recently undertaken such a study, arguing that "the number of ovules per flower (or per head in the Compositae) may be referred to as clutch size in plants, in that the development of a seed cluster from these ovules represents a discrete reproductive episode and energy commitment in time and space". (Appropriately enough, their paper is in a book commemorating David Lack: *Evolutionary Ecology*, (eds. Perrin and Stonehouse) Blackwell, 1977). One obvious problem here lies in deciding how to define 'clutch size' for plants. Levin and Turner's choice has the incidental advantage that the number of ovules per flower is relatively constant for a given species, often indeed being used by systematists as a diagnostic character. However, the net reproductive strategy for a plant also involves the total number of flowers and thence seeds, along with many other factors, which complicates any comparison with animals. Levin and Turner acknowledge this caveat, but feel their definition of plant clutch size makes for an analogy that is worth pursuing.

Levin and Turner explore the clutch size relationship within the tribe Heliantheae, which is a New World tribe of the widespread family Compositae. Their study encompasses 1,007 species from the material at the herbarium at the University of Texas at Austin. The clutch size among these

species spans a wide range, from 1 in the genera *Clibadium*, *Lagascea* and *Ichthyothere* to as much as 500 in other genera. The plants are classified according to their geography (tropics, temperate, alpine) and growth form (annual herb, perennial herb, woody shrub). The average clutch size in each category is as tabulated, with the sample size in parentheses. There are consistent patterns for the herbaceous plants, and, as expected, clutch size increases with latitude. The alpine shrubs are discrepant with the general pattern; Levin and Turner offer no explanation, but it is conceivably because this growth form is under severe stress at alpine latitudes.

Growth form	Region			Form mean
	Tropics	Temperate	Alpine	
Annual herb	39(163)	62(106)	110 (1)	49
Perennial herb	61(251)	99(161)	104(34)	78
Woody	50(249)	69 (33)	45(11)	52
Region mean	52	83	90	63

Mean clutch size as a function of geographical region and growth form, for plant species in the family Compositae. The sample sizes are given in parentheses.

As each ovule may develop into a seed after being fertilised, it is also interesting to look at the weight for single seeds and the total weight of seeds per head. Levin and Turner show that the expected negative correlation between clutch size and seed weight does exist, but that it is rather fuzzy. More significant is the total head weight, obtained by multiplying the mean clutch size by the mean seed weight. This quantity, which measures the amount of energy invested per head, averages out at 560 mg for shrubs, 350 mg for perennial herbs, and 90 mg for annual herbs. Grouping the species according to geography rather than growth form, Levin and Turner find an average head weight of 300 mg for tropical species compared with 400 mg for temperate ones. This latter result is statistically significant, and accords with our preconceptions.

As emphasised above, clutch size is only part of the overall reproductive picture, which for plants must also include seed weight, total number of seeds, dispersal mechanisms and patterns, and so on. The interplay between reproductive strategies and the shapes and sizes of seeds is discussed by Harper, Lovell and Moore (*Ann. Rev. Ecol. Syst.* 1, 327; 1970) in a masterly review that defies any glib summary. Levin and Turner survey estimates of the fraction of a plant's annual energy budget that is allocated to reproduction, and suggest for the Compositae the figure is roughly 25%

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for annual herbs, 15% for perennial herbs, and probably less than 10% for woody composites. They conclude "these general patterns suggest that, small clutch size notwithstanding, annual species of the Heliantheae will have, on average, larger reproductive efforts than perennial herbs. Small clutch size in shrubs will be associated with relatively small reproductive risks in time."

In short, Levin and Turner's study demonstrates 'clutch size' differences that are related to geography and growth form. The authors argue that "there is no reason to assume the patterns observed in the Heliantheae are unique to that tribe or the Compositae as a whole", and I echo their hope that their analysis will spark similar analyses of other plant groups. □

Synchrotron radiation at York

from G. V. Marr and I. H. Munro

SEVEN hundred delegates, over two thirds of whom were from outside the UK assembled for the fourth general conference of the European Physical Society.* In this, its tenth year of existence, the EPS took for its theme 'Trends in Physics' and 'Synchrotron Radiation' was one of the eight parallel symposia which formed the basis for a revealing insight into the varied interests of the EPS.

Synchrotron radiation and its applications was introduced and reviewed by S. P. Kapitza (Institute for Physical Problems, Academy of Science, Moscow). This general introduction to synchrotron radiation research was followed by papers on general properties and instrumentation, atoms and molecules, photoemission from solid surfaces using synchrotron and ultraviolet sources, X-ray scattering, X-ray imaging and structural studies.

For the duration of the conference, a large exhibition had been assembled by the Daresbury Laboratory of the UK Science Research Council to describe the nature, generation and exploitation of synchrotron radiation. In addition to providing information concerning the new synchrotron radiation source under construction at Daresbury contributions of material were provided from a number of European laboratories at which facilities are available for synchrotron radiation research. Representa-

tatives were present from the laboratories at Bonn and Braunschweig (West Germany), Daresbury (UK), Frascati (Italy), Hamburg (West Germany) and Orsay (France). In addition, the conference was also attended by a group of scientists from laboratories in Moscow, Novosibirsk and Yerevan in the USSR. This group visited the UK under the auspices of the USSR Commission on Synchrotron Radiation with whom in 1977 the UK Science Research Council drew up an agreement for the exchange of information and the holding of joint seminars on synchrotron radiation research.

Reviews and reports were presented at the conference on the following topics: the use of X-ray synchrotron radiation for structural research in biology; recent advances in the dynamics of photoexcited states of molecules and ions; photoemission from solids with synchrotron radiation; elastic scattering and structural applications; synchrotron radiation and X-ray crystallography; X-ray imaging, lithography, microscopy and topography; EXAFS and its use in the study of glass; atomic physics. During the meeting the contributed papers and poster contributions led to discussions of problems associated with the development of new synchrotron radiation sources and of X-ray monochromators; on the soft X-ray and VUV spectroscopy of atoms and molecules, on surface and solid state studies, on the dynamics of protein molecules, on phase transitions in biological systems, the study of clay gels, recrystallisation and grain growth and the structural studies of powdered materials. Techniques were discussed giving rapid recording of data from these very intense X-ray sources which could be used to yield structure refinement with rapidly changing sample environment.

An unusual feature—for a physics conference—was the evident importance of synchrotron radiation research in biology and in biochemistry. This was clearly demonstrated in the use of high resolution X-ray absorption spectroscopy (EXAFS) to elucidate the structure of, for example, polynuclear copper compounds which do not crystallise and of other complex metal containing proteins. Results were given on time resolved X-ray diffraction studies in the millisecond domain for muscle and on high resolution X-ray diffraction data on protein crystals, for example, tyrosyl-tRNA synthetase single crystals. Synchrotron radiation sources are intensely modulated over a wide frequency range enabling the mobility of protein molecules to be observed. Using the technique of time resolved fluorescence polarisation spectroscopy in the ultraviolet region a number of proteins were shown to

have widely differing degrees of rotational freedom of a single amino acid residue (tryptophan). The measured amplitudes and states of these motions suggested that elementary steps of functionally significant conformational changes take place in the subnanosecond time range.

The meeting revealed to the physics community at large that synchrotron radiation sources have a major contribution to make in an astonishingly wide variety of techniques and fields of scientific endeavour. Contributions indicated that developments such as undulators and the free electron laser will become increasingly important during the next decade. Indeed it is likely that future synchrotron radiation sources will be considerably different from the present generation where the radiated electromagnetic waves are the result of simple centripetal acceleration of high energy electrons confined to move on a circular orbit by a large iron core dipole magnet. The introduction of periodic magnetic structures within a straight section between conventional dipole magnets in a storage ring can result in very different forms of the emitted soft X-ray spectrum. When the vertical magnetic field alternates in direction up and down along the length of the beam orbit traversed by the electron, the oscillating electron emits a sharply peaked electromagnetic radiation spectrum. This peak occurs at a wavelength related to the product of the periodicity of the field with the square of the rest mass energy of the electron and has a width which depends inversely on the number of periods in the undulator. Increases in photon flux of up to one hundred times over that of the normal synchrotron radiation spectrum are predicted for undulator peaks in the soft X-ray region. Results reported at the conference provided details of the spectral features, angular distributions of undulating radiation and polarisation which are in basic agreement with theory, demonstrating that with further development these devices can play a significant part in the next generation of synchrotron sources.

The Synchrotron Radiation Committee of the European Science Foundation, held a two-day workshop during the EPS Conference on the specification and design of a proposed new European Synchrotron Radiation Source. This will form part of a feasibility study to be considered by the European Science Foundation late in 1979 and undulators will represent a significant part of this proposal. □

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*Held on 25–29 September at the University of York.

review article

Ogston and the development of prochirality theory

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Ogston's explanation for differentiation of identical groups in symmetrical compounds led to the important stereochemical concept of prochirality, which in turn, has made significant contributions to the understanding of enzyme reaction mechanisms.

IN 1948, Ogston postulated¹, contrary to opinions then prevailing², that identical groups in symmetrical compounds such as citrate and aminomalonate could be distinguished, one from another, in enzymatic reactions. He suggested a "three-point attachment" of a symmetrical substrate, $Ca'a''bc$ (the identical a groups are distinguished by different symbols only to facilitate discussion), to three binding sites, A' , A'' , B (which bind, respectively, a groups and the b group) on an enzyme surface. The arrangement shown for 1 (Fig. 1) is the only way in which binding can be accomplished (assuming further that binding of substrate from below the enzyme surface is not possible). It can be seen, preferably with the help of molecular models, that a' cannot be bound at site A'' , with a'' at site A' , since to do so requires that c bind at B . If $Ca'a''bc$ had been prepared in a stereo-specific manner so that only one of the a groups (for example, a') contained an isotopic label (●), and if only one of the A sites (for example, A'') was active in some catalytic process, a distinction could be made between the two a groups. As a result of these considerations, Ogston concluded "Asymmetrical occurrence of isotope in a product cannot be taken as conclusive evidence against its arising from a symmetrical precursor."

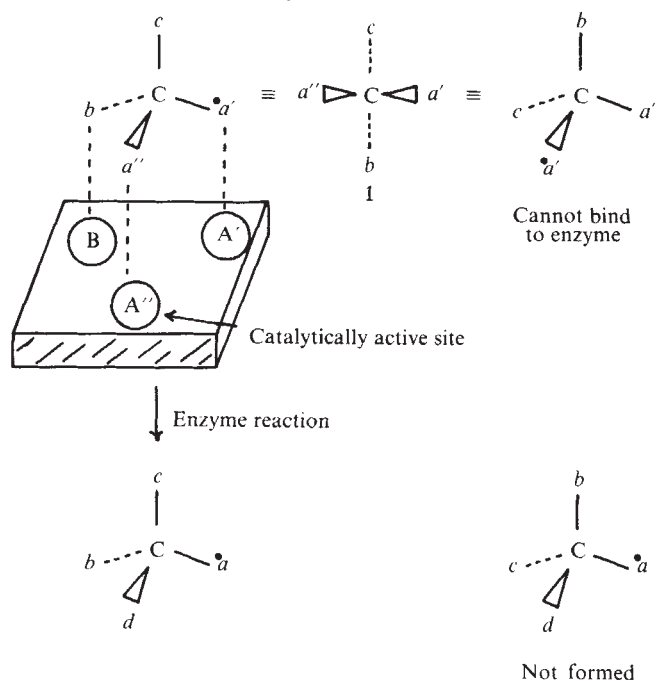
Ogston's letter stimulated lively discussion among biochemists who found it hard to accept that a symmetrical compound could behave in a non-symmetrical fashion. The difficulty people had in understanding this notion was vividly recalled by Vennesland³:

"In a mood of desperation, I sat down with Ogston's paper and took some stick and ball models to assist my thinking. Ogston's paper didn't help, though I read it several times attentively. I thought he was implying a substrate might stay stuck to the enzyme, and that did not help one bit. While I was staring bleakly at the models, they suddenly told me the answer, but not in words, just in a picture. What happened in that moment of sudden insight seemed to be that I was suddenly graced with the ability to see the asymmetric carbon atom as van't Hoff had originally seen it. The feeling I experienced was a curious combination of exhilarating, sweet relief (because there was no contradiction between the two sets of experiments) and total dismay: 'Oh you idiot, why haven't you realised that before'. There were quite a few of us who had experiences similar to mine after the appearance of Ogston's paper. Even though I felt I had grasped the point quite well I found that when I tried to transmit my comprehensions to others it was like the tower of Babel. I distinctly remember hearing myself say 'Of course I know it is symmetrical, but it isn't symmetrical'."

Despite the confusion and erroneous statements (for review see ref. 4), Ogston's conclusion was correct and it is now apparent that he had uncovered the tip of the proverbial iceberg. It seems appropriate, 30 years later, to summarise the important developments triggered by Ogston's "short but historic note"⁵.

Beginning with Pasteur's work in 1860, stereochemistry had been dominated by the object-mirror image relationship. The only requirement for chirality is that the molecular model not be superposable on its mirror image—in other words, that the molecule must possess reflectional asymmetry. It was Hirschmann who recognised that differentiation of identical groups required the presence of another type of asymmetry, namely rotational asymmetry^{4,6,7}. Hirschmann's ideas developed from a consideration of the role of diastereoisomeric transition states (or diastereoisomeric products) in the process of differentiation.

Fig. 1 The Ogston concept showing three-point attachment to an enzyme surface.



This role had been first recognised by Wilcox⁸, and can be explained as follows. Suppose a reagent reacts with $Ca'a''bc$ in a process involving one a group. Two possible transition states (or final products) will exist, one X' for reaction at a' , the other X'' for reaction at a'' . If the relationship between X' and X'' is enantiomeric, the process produces equal amounts of each since enantiomers have equal stabilities and are formed at the same rate. Hence, in such conditions a' would not be distinguished from a'' . If, on the other hand, X' and X'' are diastereoisomers, not in object-mirror image relationship, they are produced in unequal amounts since diastereoisomers have unequal stabilities or are formed at different rates. In these conditions, differentiation of the a groups could occur.

The recognition of the diastereoisomerism as the dominant factor in differentiation, either in the actual formation of a transition state or product, or even in the approach of a reagent, was a crucial step forward. The final proportions of products could depend ultimately either on the probability of successful collisions of reagent with a' or a'' (kinetic control) or on the relative stabilities or solubilities of products (thermodynamic control). Since enzymes contain many centres of chirality the diastereoisomer ratios in a transition state may become very high with almost complete formation of only one of the two possibilities.

Rotational asymmetry

After emphasising the role of the diastereoisomeric intermediates, Hirschmann⁶ provided a test to identify those structures in which differentiation of identical groups was possible (at least in principle). The role of diastereoisomeric intermediates required that "the basis for the differentiation of identical groups be sought in some structural characteristic of the substrate". The following "superposition test" was then proposed:

"A three-dimensional representation of a molecule containing two (or more) identical groups or atoms a designated as a' and a'' is moved so that the position of a'' will coincide with the original position of a' . If this can be done in such a way that the second arrangement is indistinguishable from the first, the groups a' and a'' cannot be differentiated from each other in any reaction. However, if such superposition is impossible, the groups a' and a'' can react with an asymmetric reagent at different rates".

To illustrate the Hirschmann test, I shall consider the two a groups of $Ca'a''bc$. Attempts to rotate the model (Fig. 2a) so as to position a'' on a' lead to the arrangements (Fig. 2b and c). Neither of these rotated arrangements, however, can be superposed completely on the original (Fig. 2a). Hence, a' and a'' in this structure are potentially distinguishable. Many such differentiations have been described in enzymatic reactions (for reviews, see refs 3, 4, 9-14); to quote only two examples, the protons of ethanol ($a' = a'' = H$, $b = CH_3$, $c = OH$) are distinguished by alcohol dehydrogenase, and the CH_2COOH groups of citrate ($a' = a'' = CH_2COOH$, $b = COOH$, $c = OH$) are distinguished by aconitase.

Since the symmetry operation of the Hirschmann superposition test is a rotation, compounds in which differentiation is possible have a 'rotational asymmetry'. Hirschmann also pointed out that, although in some cases, rotation of a rigid molecular structure cannot lead to superposition, this may be possible as a result of torsion about certain bonds. For example, torsion about the C-C bond of ethanol allows H' to be replaced by H'' or H''' (Fig. 3a, b or c); hence the three H atoms of the methyl group cannot be distinguished.

Fig. 2 The Hirschmann superposition test applied to $Ca'a''bc$.

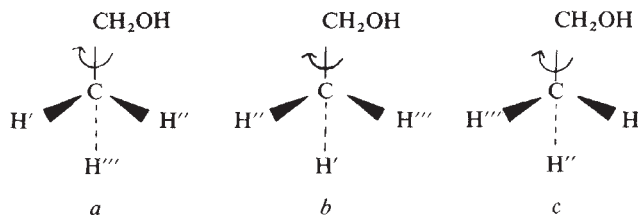
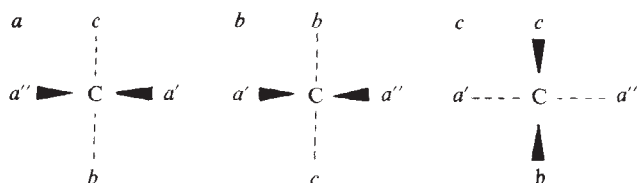


Fig. 3 Equivalence of hydrogen atoms in methyl group.

Provided that a compound has rotational asymmetry, the minimal operation which permits differentiation to occur is that of an approach of a reagent to a molecule which leads to diastereoisomeric situations for approach to a' and a'' . Clearly, a "three point combination... between the symmetrical substrate and the enzyme" is not a mandatory requirement for differentiation; Ogston has noted that his "three-point attachment" hypothesis was not meant to be taken as a literal description of the forces acting between the molecules¹⁵. Furthermore, identical groups can be differentiated by purely chemical reactions carried out in homogeneous solutions (for example, ref. 16). However, the three point attachment theory caught the imagination of biochemists and to this day is still quoted by some individuals as a requirement for differentiation of identical groups.

The concept of prochirality

Following Hirschmann's recognition of the nature of rotational asymmetry, it became desirable to have a nomenclature system for each of the two distinguishable groups in $Caabc$. Although Hirschmann⁷ had provided one solution, a more convenient system was devised by Hanson¹⁷. If one of the a groups of $Caabc$ is replaced by an achiral group, d , the product is the chiral compound, $Cabcd$. Since the substitution leads to chirality, Hanson coined the term "prochiral" for the structure $Caabc$. Thus, the central carbon atom of citrate is described as a prochiral centre. Furthermore, Hanson devised a prochirality nomenclature which relates directly to the Cahn-Ingold-Prelog system for specification of chirality. If one of the two identical ligands is considered to be preferred to the other by the sequence rule (without change of priority with respect to other ligands) and if the resultant priority order is R , then the (preferred) ligand is *pro-R* and the other *pro-S*. Prochirality assignment can be illustrated for citrate using the sequence priority and model shown in Fig. 4. However, for a complete description, the original paper or standard works should be consulted^{9,10,17}. Although the definition, concepts, and terminology for prochirality proposed by Hanson¹⁷ and elaborated on by Hirschmann and Hanson¹⁸ have come into general use in biochemistry, a somewhat different approach has been advanced by Prelog and Helmchen¹⁹. Hirschmann and Hanson have argued persuasively for the adoption of their proposals, but clearly an international agreement would be desirable²⁰.

Structural relationships

An important development has been a detailed examination of the structural relationships between groups and the molecular 'environments' within which they are located. The C-1 hydrogens of ethanol, or the two CH_2COOH groups of citrate are divided by a mirror plane. The environments of these groups at their respective prochiral centres can, therefore, be described as enantiomeric. Other environments may be described in the language of isomerism. The protons at C-3 of L-serine, for example, are not bisected by a mirror plane as is the case for the two protons just considered in ethanol. The proton environments in this case are described as diastereoisomeric. The environmental relationships of the proton at C-2 and either of the protons at C-3 of L-serine would be akin to that between constitutional isomers rather than that between stereoisomers.

Isomers are now divided into three broad groups, constitutional isomers, enantiomers, and diastereoisomers (for reviews,

see refs 21 and 22) by asking three questions regarding superposability. The three tests involve (1) comparison by symmetry operations of the first kind—rotation, torsion¹⁸, and for polymers, translation; (2) comparison of bonding (connectivity) graphs (for material on graph theory and studies of isomerism see ref. 22); and (3) comparison by symmetry operations of the second kind involving reflection operations.

Figure 5 represents this series of binary tests in which the question marks signify "Superposition? yes or no", the arrows from the sides of the diamonds, yes (acceptance), and from the bottom, no (rejection). The materially identical compounds are thus compared for superposition by symmetry operations of the first kind, Sy_I (rotation, torsion); by comparison of their bonding graphs vertex by vertex, BG; and by symmetry operations of the second kind, Sy_{II} (operations involving reflection).

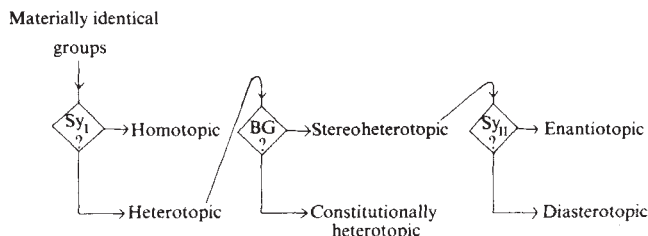


Fig. 6 Comparison of partial structures in intact molecules (or different molecules).

the model of the molecule²⁵. As groups may be compared not only within a particular molecular structure (the topic relationships) but also in partial structures, in isolation from any actual molecule^{22,25}, a further extension of the binary classification system has been proposed. Comparisons of groups in isolation are termed morphic relationships; a sequence of binary congruence tests, which parallels those for isomer and topic classifications, may be easily constructed.

Retrospect and prospect

Could the concept of prochirality have been formulated before Ogston's sudden insight in 1948? Probably not. Those working in biochemistry in the period 1930–1950 tended to have either a chemical or physiological background, and in neither case were they accustomed to think in terms of the symmetry properties of molecules. As Vennesland³ noted "Actually, anyone considering models, as Ogston obviously did, could easily grasp that compound 1 (that is, citrate with ¹¹C in one primary COOH, ¹³C in the other) has a mirror image on which it is not superimposable. The reason this had not been grasped was that the wrong answer seemed so totally and obviously right, that no one had felt it was necessary to consult models."

It has now become commonplace to determine actual configurations of labelled compounds which have rotational asymmetry. Reviews and books should be consulted for specific examples^{9–14}. One particularly interesting development has been the exploration of chirality in methyl groups containing all three isotopes of hydrogen (for review, see refs 5 and 27).

Although Ogston was primarily concerned to demolish a fallacy in the interpretation of tracer studies of intermediary metabolism, the impact of his thinking has been particularly notable in the consideration of enzyme reaction mechanisms. At a relatively unsophisticated level, the observed stereochemical course of an enzymatic reaction may allow the elimination of a proposed reaction mechanism (for examples see refs 11, 12, 14, 22 and 28). Of considerably greater interest and importance are attempts to develop more comprehensive theories, particularly by comparison of the stereochemistry of analogous enzyme reactions, and by viewing reaction stereochemistry in the context of natural selection. Hanson and Rose have suggested three important principles resulting from the natural selection for catalytic efficiency in enzymes: (1) the use of a minimal number of acidic and basic catalytic groups at the active site; (2) the maximal separation of catalytic groups and/or replacing groups; (3) the use of a minimal motion by the substrate^{11,12,14,22,28}. Further research will be needed to test and refine these challenging ideas. However, the debt to Ogston is clear.

I thank Dr B. Vennesland for permission to use the quotations from her article, Drs M. L. Bentley and I. M. Campbell for helpful discussions and Drs K. R. Hanson and H. Hirschmann for suggesting the nomenclature used in the binary diagrams.

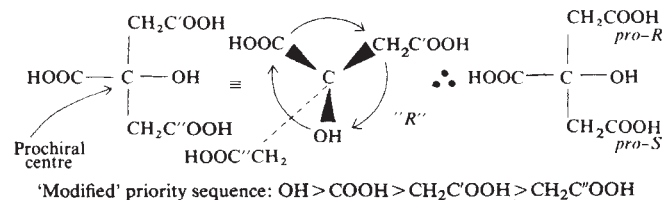
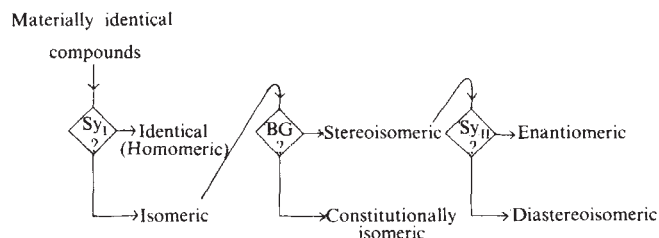


Fig. 4 Prochirality nomenclature for citrate.

Hirschmann and Hanson have used a related scheme ("topic analysis") to describe environmental relationships^{18,22} of materially identical groups within a molecule. Groups in identical superposable environments (for example, the three positions of H in CH₃—) are termed homotopic, those in non-superposable environments are heterotopic. Heterotopic (but not constitutionally heterotopic) groups are either enantiotopic or diastereotopic; these terms were first used by Mislow and Raban²³. Enantiotopic groups are those which can be interchanged only by the formal symmetry operation S_n , that is by rotation and reflection; they reside in enantiomeric environments. Enantiotopic groups are commonly encountered in biological systems (examples are the C-1 protons of ethanol and the CH₂COOH groups of citrate). The two products formed by separate substitution of enantiotopic groups, a' and a'' , by an achiral group are enantiomers. Hence a chiral reagent is required for differentiation.

Diastereotopic groups (for example, the methylene protons of citrate) are those which have the same constitution, reside in diastereoisomeric environments, and cannot be interchanged by any of the symmetry operations (rotation, reflection, rotation-reflection). There is no mirror plane between them, and if each is substituted separately by an achiral group the two products formed are diastereoisomers (this substitution test with an achiral group readily permits the distinction between enantiotopic and diastereotopic groups). In principle, diastereotopic groups can be differentiated without the intervention of any chiral agent or influence^{7,24}. A diagram for topic analysis can be constructed which relates directly to that given earlier for isomer analysis; the same symbols (Sy_I , BG, Sy_{II}) are used in the topic comparisons, the operations now being applied to materially identical groups within the molecule. It should be noted that alternative classifications are being developed; Mislow has outlined a more radical classification based on an isometry on

Fig. 5 Comparison of whole molecules—relationships between isomers.



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A. G. OGSTON COMMENTS—How did I come to make my original discovery? My undergraduate training was in pure chemistry, with a strong preference for physical chemistry, and my first research was on the conductivities of dilute electrolyte solutions in Sir Harold Hartley's laboratory in Balliol. It was my interest in electrochemistry that soon led me (opportunist that I am) into the biochemical field: first with Sir Rudolph Peters, then with E. R. Holiday and J. M. Gulland, to work on the constitutions and electrochemical properties of some biological compounds; then to work (still as a physical chemist) on immune proteins, under E. R. Holiday and J. R. Marrack, at the London Hospital; finally back to Oxford for a second degree course in Physiology and to appointment at my old College (Balliol) as Tutor, mainly teaching Physiology and Biochemistry to medical students.

Some time in 1948, when spending a spare hour looking through journals (mainly with a view to advising my pupils what to read), I came across a paper¹ which seemed to show that aminomalonic acid could not be an intermediate in the conversion of serine to glycine. I read it with interest and, initially, with consent. Suddenly, something happened. One moment I thought: 'That's neat.'; the next: 'But it's wrong.'; the next: 'Citrate!' It may have taken five seconds, perhaps less. A day or two later I sent off my letter to *Nature*².

Why did I leave it (almost^{3,4}) at that? Until I received, a few days after publication of my note, an excited letter from Sir Hans Krebs (then at Sheffield), I had little notion of the size of iceberg beneath that tip, regarding my idea as no more than an amusing piece of stereochemical logic and its consequences for asymmetric synthesis as merely an obvious corollary. And I was, by then, deeply involved with other lines of research which, if they have proved to be less sensational, have been satisfying in requiring far greater intellectual effort. 'Three-point attachment' was a gift, out of the blue, for which I have never felt able to claim much credit.

Yet I had, I suppose, unwittingly prepared myself for the three-dimensional visualisation that it required. As an undergraduate student I had enjoyed (and had been excellently taught) the intricacies of stereochemistry. During the war, on daily walks between my digs and place of work, I had amused myself by building, in my head, possible models (all quite wrong⁵) of protein structure. And I was, of course, well aware of the problem, at that time, of the place of citrate in the Krebs tricarboxylic acid cycle.

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articles

Is there a chronometer hidden deep in the Sun?

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No support is found for the conventional view of the sunspot cycle, that there exists a large random walk in the phase of the cycle. Instead, both sunspots and the [D/H] solar/terrestrial weather indicator seem to be paced by an accurate clock inside the Sun.

IT has long been believed that "the sunspot disturbances, like the eruptions of a geyser, are inherently only roughly periodic"¹. Observations show a large variation in the ~11 yr half-cycle period, "the observed intervals ranging all the way from 7.3 to 17.1 yr"¹. Kiepenheuer² has described the sunspot

cycle as follows: "It was previously believed that the sunspot cycle resulted from the superposition of different periodic cycles. . . . Since then it has become clear that the rise and fall in the number of spots is due to a number of practically independent individual processes. Thus the idea of a true periodic phenomenon was dropped in favour of the so-called 'eruption hypothesis'. On this hypothesis, each cycle represents an independent eruption of the Sun which takes about 11 yr to die down". This conception of an irregular sunspot cycle, implying a random walk in the phase of the cycle, seems to agree with the Babcock theory and with subsequent modifications of the theory. According to the Babcock theory the poloidal magnetic field remnant of one half-cycle (~11 yr) provides the seed field

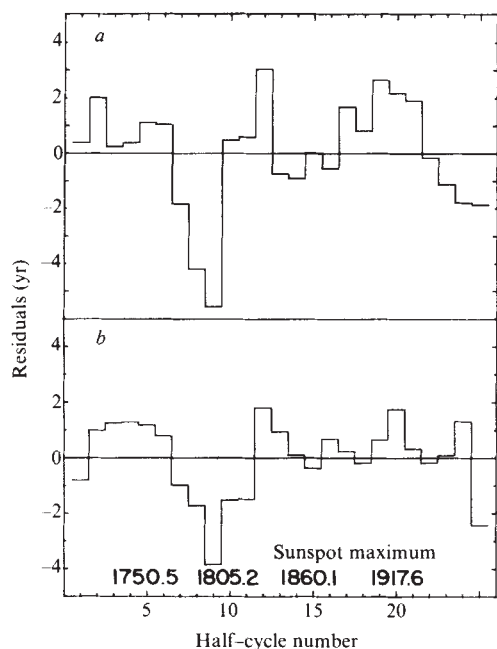


Fig. 1 *a*, Residuals (phase errors) calculated as $\delta y = y - y_0 - p(n-1)$, where y_0 and p are obtained from a least square fit of $y_0 + p(n-1)$ to y , the years of sunspot maxima. *b*, δy from equation (3).

for the generation of the toroidal magnetic field of the next half-cycle by differential winding. If, due to some vagary of the turbulent motion in the outer convection zone of the Sun, a cycle eruption should be late this lateness would be passed on to all subsequent sunspot cycles. Thus, according to the Babcock theory the sunspot cycle should undergo a random walk in phase relative to a high-precision clock.

Is a random walk in phase observed? Starting with the sunspot maximum of 1761.5 there occurred a remarkable series of three very short half-cycle periods, with an average length of 8.9 yr, after which sunspot maximum occurred ~ 5.6 yr too early. But this was followed by a 17.1-yr half-cycle that completely corrected the phase error. It is as though the Sun 'remembered' the correct phase for 27 yr and then suddenly reset the sunspot cycle (see Fig. 1*a*).

Statistics of the sunspot cycle

If the sunspot cycle were to be timed by some high- Q internal oscillator, how would we account for the remarkably large fluctuation in the intervals between sunspot maxima? One possible explanation is that the transport of magnetic field from the deep interior to the surface requires a long time and that this time interval is subject to irregularities induced by turbulence in the convective layer. In this case it might be expected that the transport interval would be lesser the greater the amount of magnetic flux floating to the surface. The phase error in the year of sunspot maximum should then correlate with the sunspot number (with a negative correlation coefficient).

To test this hypothesis the expression

$$y_0 + p(n - n_0) + r(R - \bar{R}), \text{ with } n_0 = 1 \quad (1)$$

is fitted by least squares to y_n (ref. 3), the years of sunspot maxima, starting with 1705.5 ($n = 1$). Here $n = 1, \dots, 25$, R is the sunspot number of the sunspot maximum and $\bar{R}$ is the average of the 25 values of R (ref. 3). The results are:

$$y_0 = 1,704.30 \pm 0.59; \quad p = 11.136 \pm 0.042 \\ r = -0.0397 \pm 0.0081, \quad (2)$$

with an unbiased estimate of the error in y of $\sigma_y = 1.45$ yr. The

validity of the least-square fit and the error estimates depends on the normality of the distribution of the residuals

$$\delta y = y - y_0 - p(n - n_0) - r(R - \bar{R}) \quad (3)$$

and their independence: δy is shown in Fig. 1*b*. The distribution is found to be approximately normal in form and the autocorrelation function shows only one (2σ) significant peak. The sunspot number estimates for the first few half-cycles may be substantially less reliable than the latter values. Omitting the first four half-cycles gives essentially the same results (equation (2)).

The coefficient r in equation (1) appears to be significant at the 4σ level. Hence we define a corrected year of the sunspot maximum by

$$\tilde{y} = y - r(R - \bar{R}) \quad (4)$$

This is the best estimate of the time kept by an internal clock in the Sun, if there is such a clock.

Several statistical tests can be applied to the residuals δy (equation (3)) to help decide whether or not the fluctuations in y represent the random walk in phase required by the 'eruption hypothesis'. The first test is the number of times δy changes sign. The data (Fig. 1*b*) show 10 sign changes. A Monte Carlo calculation based on a gaussian distributed random walk in phase yields 6.1 ± 2.3 crossings, but uncorrelated gaussian fluctuations yield 12.7 ± 2.5 crossings. The independent hypothesis is slightly favoured.

The second test compares two measures of the fluctuation, the mean square residual δy_n (from equation (3)) and the mean square value of $(\delta y_n - \delta y_{n-1})$. With all 25 of the sets of sunspot data, δy_n yields the ratio 0.87 for the above two measures. With the 3σ fluctuation at $n = 9$ omitted (that is, set equal to zero) the ratio is 0.65. Assuming independent, normally distributed

Table 1 Analysis of sunspot data

n_1	n_2	y_0	p	σ
1	25	$1704.30 \pm 0.55 \pm (2.9)^*$	$11.136 \pm 0.039 \pm (0.32)^*$	1.415 (1.479)
1	13	$1704.72 \pm 0.90 \pm (2.4)$	$11.041 \pm 0.127 \pm (0.49)$	1.710 (1.600)
14	25	$1705.52 \pm 1.66 \pm (6.6)$	$11.079 \pm 0.088 \pm (0.46)$	1.055 (1.456)
5	25	$1703.61 \pm 0.79 \pm (3.4)$	$11.176 \pm 0.052 \pm (0.38)$	1.443 (1.572)
5	15	$1703.47 \pm 1.57 \pm (3.5)$	$11.178 \pm 0.165 \pm (0.56)$	1.728 (1.692)
15	25	$1705.95 \pm 2.03 \pm (7.7)$	$11.058 \pm 0.105 \pm (0.51)$	1.103 (1.526)

* Errors in parentheses assume random walk in phase.

fluctuations in y_n , the ratio of the expectation values of the two above mean squares is $(N^2 - 1)/2(N^2 + 2N + 3)$, which for $N = 25$ is 0.46. Assuming a random walk in $\tilde{y}$, namely that the error in $\tilde{y}_n$ is $\sum_{m=1}^n W_m$ with W_m normally distributed and independent and with a uniform variance, the ratio of the expectation values of the two mean squares is $(N + 2)(N^2 - 1)/3(5N^2 + 6N - 3)$, which for $N = 25$ is 1.72. Again the independent fluctuation hypothesis is favoured.

The third test is more powerful. It depends on the fact that a random walk in phase implies a large expected statistical inaccuracy in the values of y_0 and p obtained from the least-square fit

$$y_0 + p(n - n_0) \rightarrow \tilde{y} \quad (5)$$

The sunspot data can be analysed as a whole and also in pieces. Substantial inconsistencies would be expected in the results if a random walk in phase occurred. The results are given in Table 1. The more unreliable data are dropped in the last three rows of Table 1.

In all columns of Table 1 the first error (without parentheses) assumes independent fluctuations and the second (with parentheses) assumes a random walk. The standard deviation of the error steps W (last column, parentheses) is chosen to give the same expected r.m.s. value for $(\delta y_{n+1} - \delta y_n)$ as is obtained from the data residuals, equation (3). For independent fluctuations, σ

(last column, without parentheses) is the unbiased estimate of the standard deviation of the error in y , that is,

$$\sigma^2 = N \delta y^2 / (N - 2) \quad (6)$$

For independent fluctuations the expected variances of the errors in p and y_0 are respectively

$$\sigma_p^2 = \sigma^2 / N(n - \bar{n})^2 = 12\sigma^2 / N(N^2 - 1) \quad (7)$$

$$\sigma_{y_0}^2 = \sigma_p^2 (n - n_0)^2 = 2\sigma^2 (2N - 1) / N(N + 1) \quad (8)$$

(assuming $n_0 = 1$).

For random walk fluctuations the corresponding expectation values are

$$\sigma_p^2 = 1.2(N^2 + 1)\sigma^2 / N(N^2 - 1) \quad (9)$$

and

$$\begin{aligned} \sigma_{y_0}^2 &= [(N + 1)(2N + 1) / 6N - (n - n_0) \\ &\quad + 6(N^2 + 1)(n - n_0)^2 / 5N(N^2 - 1)] \sigma^2 \quad (10) \\ \sigma_{y_0}^2 &= 2\sigma^2 (N^3 + 4N^2 + 11N - 1) / 15N(N + 1) \end{aligned}$$

(assuming $n_0 = 1$).

Table 1 shows that the agreement among the values of y_0 and p is very good, better than would be expected if there were a random walk in phase. But it should be emphasised that all the determinations of y_0 and p in Table 1 are not independent. Also, in line 3, σ_{y_0} is the expected error due to the data (14, 25). But the random-walk phase error at the termination of (1, 13) is the starting error for (14, 25).

A fourth test of the random-walk hypothesis is provided by the unbiased estimate of σ^2 , given by equation (6). For a data string broken into m equal pieces the average of the m values of σ^2 should closely approximate σ^2 for the unbroken string, assuming independent fluctuations. With a random walk, the expected mean variance is a factor $(N + 2m) / m(N + 2)$ smaller. The r.m.s. value of σ from lines 2 and 3 of Table 1 is 1.421 yr, to be compared with 1.415 yr. From the 5th and 6th lines 1.450 yr is obtained, to be compared with 1.443. With random walk errors, 1.044 yr and 1.066 yr would be expected.

All four tests tend to support the independent fluctuation hypothesis, but this support for the 'solar clock' hypothesis is not decisive as the data sample is small and the results could represent a statistical fluke.

Pre-Maunder-minimum sunspot cycle

As a result of Eddy's work⁴ it now seems to be well established that very few sunspots were observed from 1645 until 1700. An interesting question concerns the phase and frequency of the pre-minimum sunspot cycle. Was there an internal clock running while the sunspot cycle was substantially turned off?

The telescopic observations during the early seventeenth century were too few to answer this question conclusively. Eddy has found evidence for three sunspot maxima between 1610 and 1645. Taking the sunspot numbers estimated by Eddy at face value, and assuming that $p = 11.136$, gives $y_0 = 1702.2$, with the correction in equation (4). If it is assumed that R was underestimated by a factor of 2, $y_0 = 1703.7$ is obtained. The agreement of these values with y_0 on the first line of Table 1 may mean that a solar clock was running while the surface indications of the cycle were substantially switched off, but the data are too few to provide strong support for this conclusion.

A very long, reliable data base is preferable for an independent analysis of the pre-Maunder-minimum sunspot cycle. The naked-eye sunspot observations of the past 2,000 yr provide one possibility for such an analysis⁵. Unfortunately these large sunspot groups do not always occur at the time of the sunspot maximum and the resulting scatter in epoch seems to be so large as to make these data worthless. Another approach is to use the historical accounts of auroras which are known to correlate with the sunspot cycle. Again there seems to be a large phase uncertainty. There is also an additional uncertainty, that of identification. "Auroras" in ancient Far Eastern accounts are

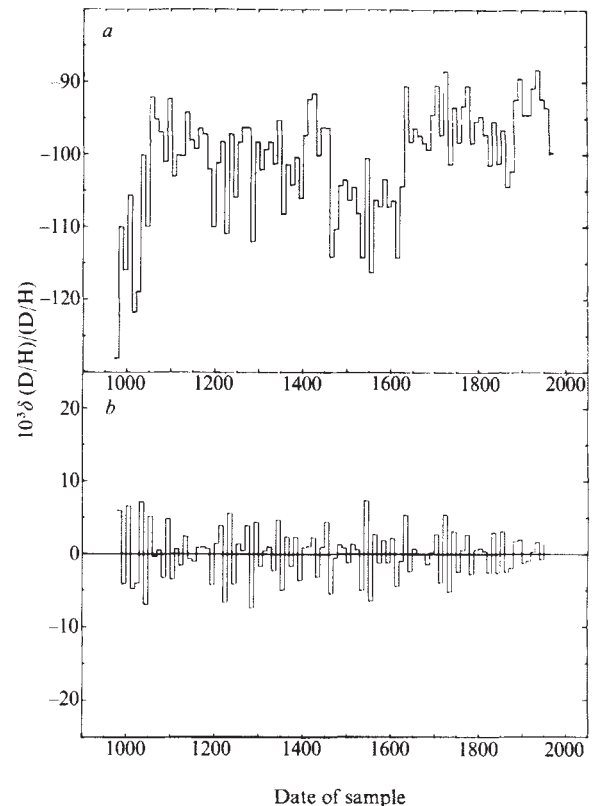


Fig. 2 *a*, Epstein-Yapp⁷ [D/H] bristle cone pine data, *D*. *b*, Epstein-Yapp⁷ [D/H] data filtered by subtracting a moving mean $\bar{D}_n = (D_{n-1} + 2D_n + D_{n+1})/4$ to give $\delta D_n = D_n - \bar{D}_n$.

described as "dark vapours", "white vapours", "greyish clouds", and so on.

The long-debated hypothetical relationship between sunspots and weather might provide the necessary data. This has been a controversial matter: some atmospheric scientists say that the miniscule change in the solar spectrum associated with the sunspot cycle should not have a substantial effect on the weather, others find evidence for a 22-yr cycle in the weather, and some astrophysicists say that a significant variation in the solar luminosity with the sunspot cycle could not occur. In discussing the 22-yr period in the drought record for the High Plains, Roberts⁵ remarked in 1973 that "it will be extremely interesting to see what happens in this region in the period 1974 to 1978". Perhaps this anticipation of the drought of 1976-77 should have been taken seriously. Such a prediction could reasonably have been based on the statistics of previous droughts. The drought years of the nineteenth and twentieth centuries in the High Plains seem to be rather well-established and are: $y = 1823, 1843, 1868, 1890, 1913, 1934$ and 1954 (ref. 6). A least square fit of the form of equation (5) gives

$$\begin{aligned} y_0 &= 1889.3 \pm 0.6 \text{ y} \\ p &= 22.14 \pm 0.28 \text{ y} \\ \sigma_y &= 1.474 \text{ y.} \end{aligned} \quad (11)$$

The expected epoch of the 1975-76 drought is $y = 1977.9 \pm 1.7$. The value of p in equation (11) is in good agreement with twice the values of p in Table 1. If we assume that these periods are the same and adopt the more accurate value $p = 2 \times 11.136$ yr as the cycle period (Table 1, line 1), we obtain $y_0 = 1889.3 \pm 0.5$ and $\sigma_y = 1.375$. The drought epoch closest to 1705 is 1711.1, at the time of the sunspot minimum.

The $\delta[D/H]$ indicator

The data from which conclusions can be drawn concerning the phase and frequency stability of the pre-Maunder sunspot cycle are given in Fig. 3a of ref. 7. This shows the sequential variations in the deuterium/hydrogen ($[D/H]$) ratio of a set of 10-yr samples of cellulose nitrate obtained from two bristle cone pines that cover 1,000 yr of time without gaps. A 60-yr interval of the 1,000-yr span overlaps data from the two trees and gives consistent results⁸. For conciseness I shall use D to designate the $\delta[D/H]$ indicator, the fractional variation in the $[D/H]$ ratio relative to $[D/H]$ of a standard mean ocean water. Expressed in parts per thousand, this indicator is $D = 10^3 \delta[D/H]/[D/H]$.

Yapp and Epstein⁹ have noted that the $[D/H]$ ratio found in precipitated water varies with both atmospheric temperature and mean surface oceanic temperature, and they have demonstrated that such a variation shows up in the $[D/H]$ ratio found in the cellulose of plants that incorporate the precipitated water during their growth. The variation of D with atmospheric temperature, expressed as a mean slope is $\langle dD/dT \rangle \approx 5.4 \text{ deg}^{-1}$ (see Fig. 10 of ref. 9). Epstein and Yapp⁷ have demonstrated a connection between their indicator and the weather. The missing element is a convincing argument that this indicator is related to the solar cycle.

Figure 2a is Fig. 3a of ref. 7 replotted. The approximately 20-yr period is clearly visible. The power spectrum of D shows a strong peak with a 22 yr period (Fig. 4 of ref. 7). This analysis is repeated here at a higher resolution and after filtering D by subtracting a moving mean $\bar{D}$. δD is shown in Fig. 2b. Figure 3 shows the power spectrum of δD . The principal peak occurs at an interpolated frequency of 0.04472 yr^{-1} and the corresponding half-period is 11.181 yr. The agreement with p in the first line of Table 1 is excellent. This close agreement and the narrowness of the peak in Fig. 3 provide substantial support for the argument that there is a causal relationship between the sunspot cycle and the $[D/H]$ indicator, and therefore with the weather. The spectral peak has the same width at half maximum as that of a sinusoidal oscillation observed for 1,000 yr. The existence of a purely terrestrial meteorological resonance of such high Q seems very unlikely. For the resonance frequency to accidentally agree with the sunspot cycle frequency with a 1 σ accuracy of 0.5% seems equally unlikely.

Because of aliasing by the 10-yr sampling period the above interpretation of the frequency of the spectral peak is not unique. However, filtering by the 10-yr average substantially eliminates all but one of the alternative frequencies (0.05528 yr^{-1}) which does not seem to be the correct interpretation.

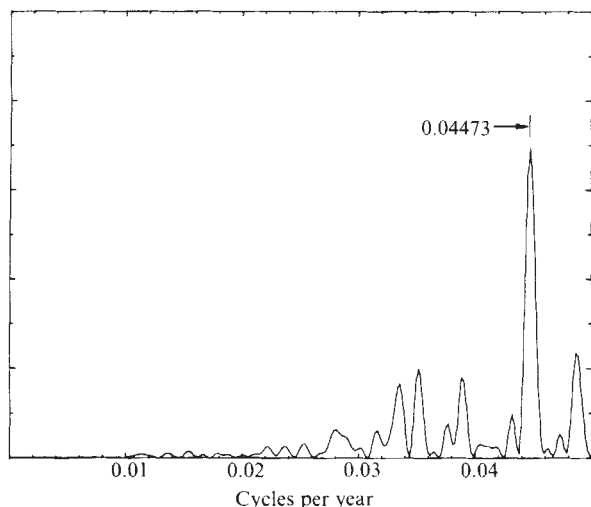


Fig. 3 The power spectrum of δD of Fig. 2. the main peak occurs at the solar cycle frequency (given by the top line of Table 1) to the statistical accuracy of the latter.

Additional support for the assumption that the Epstein–Yapp data provide information about the solar cycle is found in the apparent “finger-print” of the Maunder minimum in the data. These data (Fig. 2a) show a pronounced step upward in D after 1625, shortly before the start of the Maunder minimum. The cause of the step is unknown but cannot be due to the change in sample source (change in trees); conceivably it might be related to the Maunder minimum (S. Epstein, personal communication). At 1645 there occurs the start of a 50-yr interval (during the Maunder minimum) with very little variation in D , the only interval of that type in the 1,000-yr span. This quiet interval ends in 1695, just before the return of normal sunspot activity.

We shall tentatively assume a causal relationship between the solar cycle and the weather and treat the Epstein–Yapp data as a solar-cycle indicator. It should be emphasised that this does not imply that the sunspots or other surface manifestations of solar activity are directly responsible for the variation in $[D/H]$. And the phase of the $[D/H]$ variation need not correspond with the phase of the spot eruption. As noted above the prominent spectral peak found in Fig. 3 is characteristic of a sinusoidal oscillation. The small, narrow secondary peaks of various heights are characteristic of a contamination of the signal by gaussian noise. If random-walk fluctuations in phase should occur the spectral character of the signal would be expected to be significantly different from the peak in Fig. 3. A complex multiple peak resonance with several peaks rising out of a broad low base is normally expected. This is illustrated in Fig. 4.

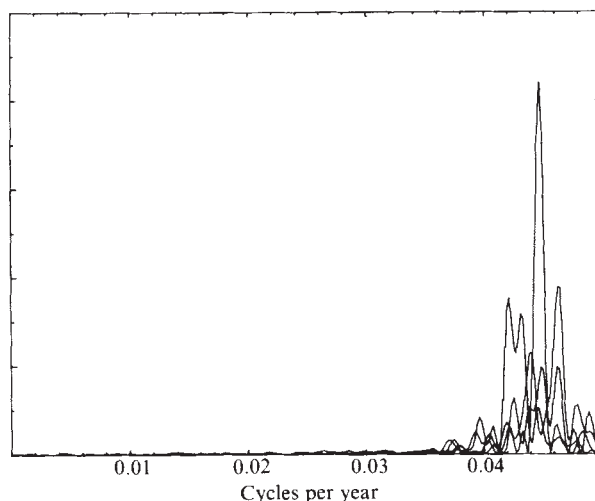


Fig. 4 The power spectra of ‘dummy data’, computed assuming a constant amplitude oscillation of frequency 0.04473 yr^{-1} and a random walk in phase. The variance of the random-walk steps $(1.402)^2/10 \text{ yr}$ is equivalent to the random-walk variance $(1.479)^2$ in Table 1. Four randomly chosen examples are superimposed.

We can now examine the Epstein–Yapp data for frequency and phase stability. From equations (7)–(10) the difference between the errors expected under the eruption hypothesis and the solar clock hypothesis are more extreme the longer the data span, the error ratio being approximately proportional to the length of the span. To examine the frequency stability the frequency $\nu = \omega/2\pi$ and the coefficients c_0 , c_1 and c_2 are adjusted by least squares in the fit

$$c_0 + c_1 \cos \omega(t - t_0) + c_2 \sin \omega(t - t_0) \rightarrow \delta D \quad (12)$$

(assuming independent fluctuations) where $t_0 = 1705$. This gives $\nu = 0.044722 \pm 0.000082 \text{ yr}^{-1}$ for the full data set ($n_1 = 1$, $n_2 = 98$) and a corresponding half-period of $11.180 \pm 0.021 \text{ yr}$. (Compare with Table 1, first line.) For the first half of the data ($n_1 = 1$, $n_2 = 49$) we obtain $\nu = 0.04500 \pm 0.00019 \text{ yr}^{-1}$ and for the second half $\nu = 0.04429 \pm 0.00023 \text{ yr}^{-1}$. For the intervals (1, 25), (25, 49), (50, 74) and (74, 98) the frequencies are 0.04443 ± 0.00062 , 0.04585 ± 0.00040 , 0.04455 ± 0.00053 and $0.04415 \pm 0.00070 \text{ yr}^{-1}$ respectively.

Table 2 Analysis of [D/H] data

n_1	n_2	c_1	c_2	σ_D	δ
1	98	1.11 ± 0.39	-2.47 ± 0.38	2.687	$-66^\circ \pm 8^\circ$
1	49	1.20 ± 0.62	-3.02 ± 0.60	3.011	$-68^\circ \pm 11^\circ$
50	98	1.00 ± 0.48	-1.91 ± 0.47	2.344	$-62^\circ \pm 13^\circ$
24	73	1.01 ± 0.53	-2.84 ± 0.55	2.683	$-70^\circ \pm 10^\circ$
1	33	1.85 ± 0.85	-3.06 ± 0.79	3.308	$-59^\circ \pm 13^\circ$
34	66	0.27 ± 0.59	-3.18 ± 0.64	2.476	$-85^\circ \pm 11^\circ$
66	98	1.52 ± 0.53	-1.46 ± 0.49	2.059	$-44^\circ \pm 14^\circ$
1	25	1.99 ± 1.01	-2.77 ± 0.96	3.412	$-54^\circ \pm 16^\circ$
25	49	0.87 ± 0.80	-3.31 ± 0.83	3.425	$-75^\circ \pm 13^\circ$
50	74	0.93 ± 0.69	-2.61 ± 0.73	2.774	$-70^\circ \pm 15^\circ$
74	98	1.40 ± 0.66	-1.32 ± 0.61	1.926	$-43^\circ \pm 18^\circ$

Adopted frequency, $\nu = 0.044722 \text{ yr}^{-1}$.

The agreement of the various values of ν with each other is much better than would be expected with a random walk in phase. Despite the slightly high frequency for the (25, 49) interval, the frequency determinations are internally consistent assuming independent fluctuations. To test for phase stability we adopt $\nu = 0.044722 \text{ yr}^{-1}$ and obtain the results shown in Table 2. In Table 2 the last column is the phase lag (in degrees) of the oscillation (relative to the time zero 1705). (A phase angle of $\delta = -66^\circ$ corresponds to a maximum in δD at 1701.) The amount of stability of phase shown in Table 2 is consistent with uncorrelated fluctuations and is greater than that expected under the random-walk hypothesis.

The Epstein-Yapp data are consistent with the hypothesis that the solar cycle persisted through the Maunder minimum. Additional support for this conclusion is found by Mitchell and Stockton in the tree-ring data¹⁰. It is found that the power spectra of indices derived from the variations in the widths of successive tree rings exhibit strong but broad 22-yr peaks which seem to be due to the solar cycle¹¹.

Conclusions

From this it may be concluded that: (1) the sunspot cycle shows no statistical indication of a random walk in phase; (2) it appears to exhibit large fluctuations in phase relative to some internal oscillator in the Sun; (3) the large magnitude of some of the phase errors (as great as 5.6 yr) suggests that there may be a long delay in the transport of magnetic field to the surface of the Sun from the interior; (4) there is a good correlation of this delay interval with the sunspot number and presumably with the amount of magnetic flux; (5) there are several indications that the solar cycle persists through the Maunder minimum. The signal strength and the precision of the 22.36 ± 0.04 -yr period seen in the Epstein-Yapp [D/H] data, the narrowness of the spectral peak and the tight agreement with the 22.27 ± 0.08 -yr magnetic sunspot period strongly support the hypothetical connection between weather and the solar cycle.

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A Glacial Mediterranean

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Glacial Mediterranean palaeo-oceanography has been reconstructed from planktonic foraminifers once living in this small ocean. Inferred sea surface temperatures ranged from 13 °C in the Alboran Sea to 18 °C in the Levantine Sea during the winter and from 19 to 26 °C respectively during the summer. An influx of cool, fresh surface water from the Aegean Sea disturbed this gradient in the eastern Mediterranean.

THE Mediterranean is a small elongate ocean basin separating dry, arid and hot North Africa from humid to semiarid, temperate to subtropical Europe, and is therefore a sensitive climatic boundary at present. As a marine basin it is subdivided by shallow sills into a series of deep troughs (Fig. 1), and exchanges its water masses with the Atlantic Ocean through the Strait of Gibraltar, to a minor degree with the Black Sea through the Bosphorus and with the Red Sea through the Suez Canal. During the Quaternary the pathways between the various basins of the Mediterranean, and the connection to the Atlantic, must frequently have been much narrower and more restricted than at present due to the eustatic lowering of the Glacial sea levels at the same time as the Black Sea became a fresh water or saline lake¹. The mode of water exchange through the Strait of Gibraltar is largely climatically controlled²: North-east Atlantic surface waters which are relatively cool and of normal oceanic salinity, flow into the Mediterranean where evaporation exceeds precipitation and fresh water input from the various rivers. The

excess inflow is partly balanced by outflowing Mediterranean subsurface waters which are dense due to their high salinity and relatively warm. They spill over the sill at Gibraltar (330 m), sink to a water depth interval³ of ~500–1,500 m and can be traced as a separate intermediate water mass over wide regions of the Atlantic Ocean. Any change of this balance which might have occurred during the climatic pulsations of the late Quaternary, must have had important implications for the vertical water mass structure of the entire North Atlantic Ocean. There is an intense dispute about the possibility of a reversed current regime in the Strait of Gibraltar during Glacial episodes^{4,5}.

Mediterranean planktonic foraminifers

Here I use planktonic foraminiferal assemblages to reconstruct the Glacial palaeo-oceanography of the Mediterranean. Samples have been dated by various methods (litho- and biostratigraphy, oxygen isotope ratios, radiocarbon dating) and will be discussed elsewhere together with a detailed account of the foraminiferal data. The most important recent advance in the stratigraphy of late Quaternary Mediterranean pelagic sediments, which enabled the correlation of these sediments to the contemporaneous record in other oceans⁶, is the recognition of the coincidence of the elevated oxygen isotope ratios marking the last Glacial maximum with the occurrence of the coolest planktonic foraminiferal faunas^{7–9}. Glacial core levels have been sampled and planktonic foraminiferal taphocoenoses have been counted at all locations (Fig. 2a and b) except the position off Israel where published data have been used¹⁰. Faunal counts

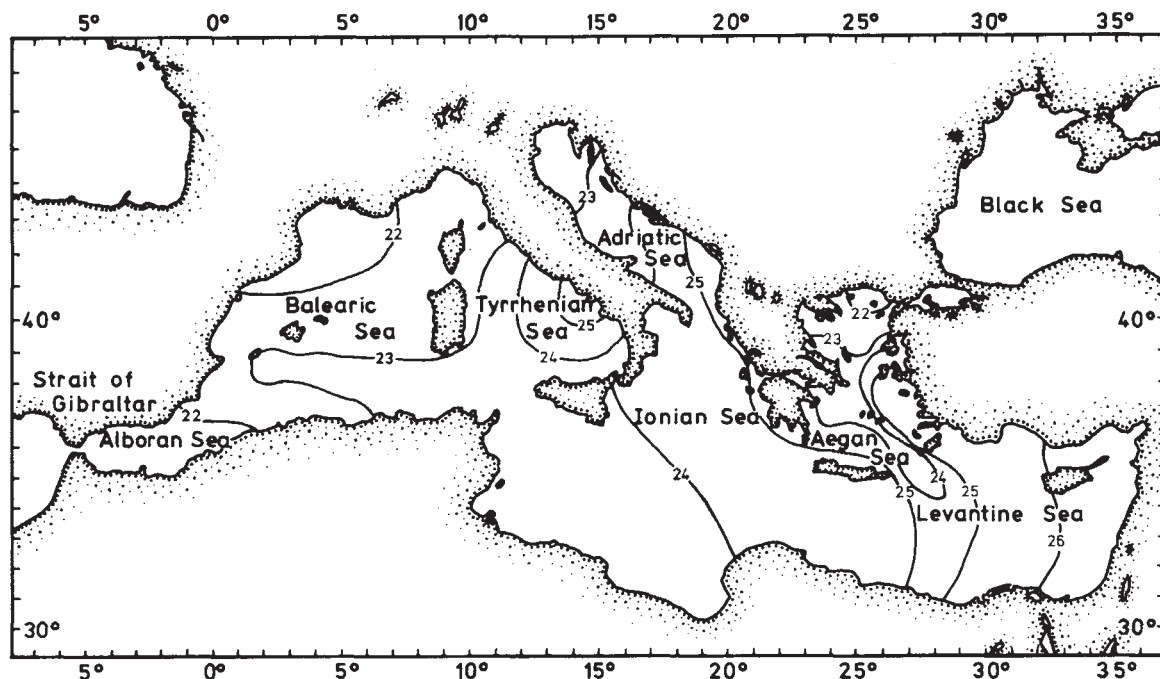


Fig. 1 Modern summer (August) sea surface temperatures in the Mediterranean after Bruns⁴⁰ and names of major basins as referred to in the text.

and palaeo-oceanographic estimates of 33 locations provide the data base for this reconstruction of the Glacial Mediterranean.

Planktonic foraminifers and other planktonic fossils have been used successfully in many parts of the world ocean¹¹ for palaeo-oceanographic reconstructions, but these methods have encountered difficulties when applied to regions close to the continents¹² and in marginal basins. The late Quaternary foraminiferal faunas from the East and West Mediterranean have frequently been described since the Swedish Deep Sea Expedition assembled the first substantial series of sediment cores from this region^{13,14}. However, most of these studies¹⁵ have looked at the late Quaternary fluctuations as a series of events affecting the depositional regime at a particular core location. In this study, emphasis is placed on drawing a regional, quasi-synoptic picture of the palaeo-oceanography of the entire Mediterranean Sea during the last Glacial by collecting as many samples as possible from cores where I could confidently identify this stratigraphic interval, and by using Glacial planktonic foraminiferal faunas to define the distribution of important surface water masses during the 18,000 yr BP time slice.

Atlantic surface waters transport planktonic foraminifers into the Mediterranean¹⁶, where rich faunas dwell in the surface water masses above all the central troughs. Planktonic foraminifers, which inhabit the modern oceans from polar to tropical latitudes¹⁷, are confined to water masses with a relatively narrow range of salinities. They cannot therefore survive or establish autochthonous faunas in marginal seas such as the North Sea or Baltic Sea^{18,19} where the seawater is considerably less saline than in the open ocean, or in the Persian Gulf, where high salinities prevent the establishment of autochthonous faunas (although living planktonic foraminifers drift into this marginal basin from the Indian Ocean)^{20,21}. It is therefore important that planktonic foraminifers inhabited the Mediterranean throughout the late Quaternary^{22,23} despite evidence from the surrounding land masses suggesting major climatic fluctuations in this region^{24,25}. The common presence of these fossils in the Glacial and Interglacial Mediterranean sediments, despite the periodically restricted pathways between the North-east Atlantic and the Alboran Sea, and between the west and east basins is remarkable because it implies that it was the Mediterranean sea surface temperature regime that responded to these climatic fluctuations rather than the salinities, although

detailed investigations of the oxygen isotope ratios of planktonic foraminifers also suggest important variations of sea surface salinities²⁶. The western basins are now populated by tropical, subtropical and temperate faunas while tropical faunas prevail in the East Mediterranean. A typical warm water representative of these faunas is *Globigerinoides ruber* which is a frequent component of Tyrrhenian Sea, Adriatic Sea and East Mediterranean surface sediments^{27,28}. During the last Glacial this and other tropical species were restricted almost entirely to the Ionian and Levantine Seas^{10,13,14,22}.

Although the Balearic Sea is situated just south of a region of major alpine glaciation during the last ice age, and although we know that the Bay of Biscay was filled with polar water masses during this period^{12,29}, it was surprising that the typical planktonic foraminiferal marker species of polar water masses, the left-coiling phenotype of *Globobulimina pachyderma*, never became abundant in the Mediterranean except in a small area of the Tyrrhenian basin. In the Glacial North-east Atlantic, water masses with this species have been traced as part of the eastern boundary current system along the Iberian continental margin, but they do not seem to have protruded southwards beyond the south-west tip of the Iberian peninsula. Cool to temperate species inhabited the Glacial West Mediterranean with species such as *Globigerina bulloides*, *G. quinqueloba*, *G. pachyderma* (right) and *Globorotalia inflata* being typical faunal components. Note that some of these species have also occurred in considerable abundance in the northern parts of the Levantine and Ionian Seas as well as in the Adriatic³⁰.

Glacial sea-surface temperatures in the Mediterranean

Although Mediterranean faunas posed a considerable problem in early attempts at synoptic palaeo-oceanographic reconstructions, my studies apparently revealed many similarities to equivalent Atlantic faunas. I have therefore attempted to explain the composition of the Mediterranean faunas statistically in terms of North Atlantic assemblages and to apply transfer functions³¹ which had been developed to describe quantitatively the North-east Atlantic late Quaternary palaeo-oceanography¹², to Glacial Mediterranean planktonic foraminiferal

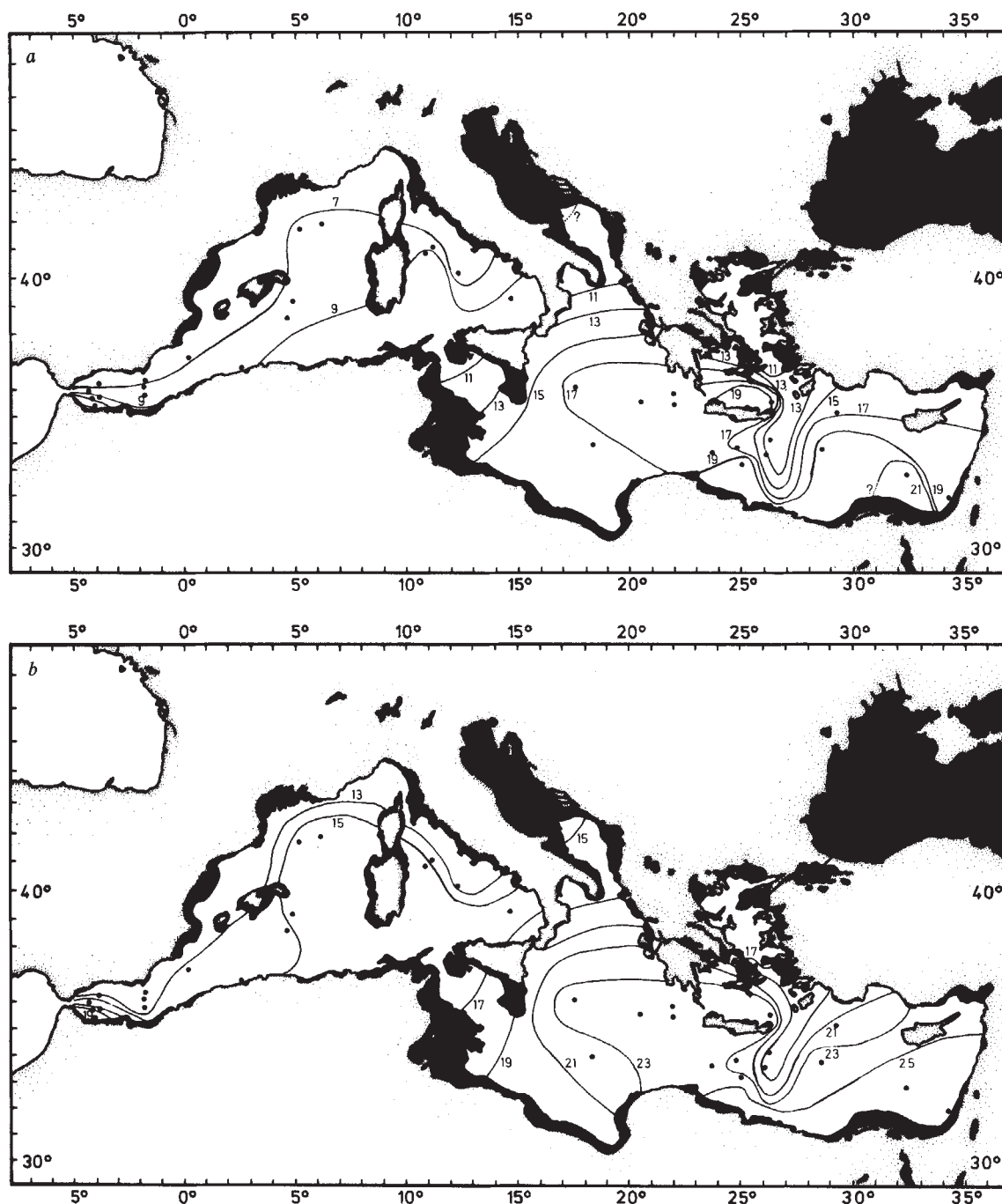


Fig. 2 Sea-surface temperatures in the Mediterranean during the peak of the last Glacial ~18,000 yr ago. *a*, During the Glacial winter (February). *b*, During the Glacial summer (August). Dots mark the positions from where Glacial core levels have been obtained. The blackened areas mark schematically changes of the Mediterranean coast lines due to the lowered Glacial sea levels.

shell assemblages. For this the data base which had been developed for the North Atlantic³², has had to be modified by selecting the data from the North-east Atlantic and by adding a considerable number of sample points from the Bay of Biscay and from the Ibero-Moroccan continental margins¹². These modifications were intended to recognise and allow for the fact that it is the surface waters of the North-east Atlantic eastern boundary current system that are diverted into the Mediterranean. Downcore studies suggest there is good reason to believe that the Glacial mode of water exchange between the Mediterranean and the North-east Atlantic resembled the modern current regime in the Strait of Gibraltar⁵ when the >300 m deep strait was probably only half as deep than at

present³³. The Mediterranean can also be considered as a small ocean basin with plankton assemblages slightly different from those of the open pelagic environment, because they are affected by the hydrography of epicontinental water masses which are never very far away in this small ocean.

Otherwise the evaluation of the micropalaeontological data has followed established procedures³¹ which allowed me to estimate quantitative values for past oceanographic variables. They combine Q-mode factor analysis with regression and classification analysis. The Q-mode factor analysis with the program CABFAC³⁴ produces two matrices. The varimax factor matrix defines the importance of North-east Atlantic planktonic foraminiferal assemblages = factors at each sample

location¹², a matrix of factor scores indicates the contribution of each category = planktonic foraminiferal species to each factor. The relationship among assemblages and hydrographic variables has been established by multiple regression analysis, in this case North-east Atlantic summer (August) and winter (February) sea-surface temperatures. The resulting transfer functions have recently been used to reconstruct the Glacial North-east Atlantic¹². In this study classification analysis has been applied to project Glacial Mediterranean downcore samples into the model derived from the modern North-east Atlantic. This step allows the factor loadings to be estimated on the basis of the predefined matrix of factor scores in each of the Mediterranean samples. Finally, these estimated factor loadings have been fed into the equations obtained by regression analysis, to calculate values of summer and winter sea-surface temperatures in the Glacial Mediterranean.

The degree to which planktonic foraminiferal faunas in each sample are explained by the Q-mode factor model, is expressed as communality both in factor and classification analysis. Thus, the calculated communalities of the Glacial Mediterranean faunas can tell how successfully they are explained by the North-east Atlantic surface assemblages. Several stations have been found where the faunas have not been explained satisfactorily by the model applied, as indicated by low communalities (cut off point in general at 0.7). These stations are located mainly in the marginal basins along the northern shores of the Mediterranean, for example in the Adriatic Sea. I conclude, therefore, that these faunas do not have an analogue in the modern North-east Atlantic. Other samples with low communalities have been observed in regions where frequent turbidites (for example, in the abyssal plains) suggest the contribution of displaced sediment components. Samples with low communalities have therefore been eliminated from further analysis.

This attempt to reconstruct the palaeo-oceanography of the Glacial Mediterranean reveals several other deficiencies. Although sea-surface temperatures have been compared here with Atlantic data, other hydrographic variables might have been more appropriate. Distortions of the oxygen isotope ratios of planktonic foraminiferal shell material suggest important changes of sea surface salinities²⁶. The eastern Mediterranean is today characterised by seasonal differences of temperature which are higher than those in the North-east Atlantic, because the influence of the continental climate over the adjacent land masses has an important impact on the temperature regime. The model applied here might therefore be incapable of generating sufficiently high seasonalities for the Glacial Eastern Mediterranean.

However, there are enough cores whose faunas are explained very well by the above described model (Fig. 2). The factor loadings which have been estimated in the classification analysis-step, reveal a simple regional distribution pattern of planktonic foraminiferal assemblages in the Glacial Mediterranean.

Two relatively cool assemblages are concentrated in the north-west basins, an intermediate one occurs as a belt across the central part while the tropical assemblage is confined to its south-west region. Polar assemblages are virtually missing from the entire basin.

The results of attempts to contour the temperature estimates for the Glacial winter (February) and summer (August) are given in Fig. 2a and b. The temperature range spanned from 7 °C in the north-west basin to >20 °C in the south-east basin during the Glacial winter. While the temperature increased eastwards in a fairly regular fashion in the Balearic basin, the pool of warm surface waters in the Ionian and Levantine basins reveals a considerable structure because of the disruptive intrusion of a cool surface water mass from the Aegean Sea. During the summer the entire Mediterranean apparently warmed up considerably, but the basic pattern of surface-water temperatures was still preserved. The steepest temperature gradients are found close to the connection between the East and West Mediterranean basins³⁵, but the incursion of the cool surface water from the Aegean basin is also visible during the Glacial summer. Comparison between the modern (Fig. 1) and Glacial (Fig. 2b) sea-surface temperature shows how and where the most drastic temperature changes from the Glacial to the modern situation have occurred. The similarity of the pattern, although not of the absolute temperature values south of the Aegean Sea, is particularly noteworthy. The temperatures in the Glacial Alboran Sea compare well to those of the adjacent North-east Atlantic. The Bay of Biscay¹² was probably a few degrees colder than the North-west Mediterranean during Glacial times. Although we know that ice-rafted debris can be traced in the Glacial levels of cores along the north-west African continental margin as far as South Morocco³⁶, coarse ice-rafted terrigenous detritus has not been found in the corresponding samples from the Western Mediterranean. The higher sea-surface temperatures in the Glacial Balearic basin are also supported by the virtual absence of the polar planktonic foraminifer *G. pachyderma*.

A source for the water mass protruding from the Aegean into the Levantine basin deserves further attention. Previously it had been assumed that the fresh water influx was due to high discharge rates of the Nile river⁹, but the pattern which has now been established by means of foraminiferal studies, strongly suggests a northern source^{37,38}. It is commonly believed that the land areas north of the Glacial Mediterranean were much drier than at present^{24,39}. If this is so, the source for this important influx of cool and probably comparatively fresh water²⁶ into the Aegean Sea has to be sought in the hinterland of the Glacial Black Sea which as a lake was overflowing into the Mediterranean^{37,38}.

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The regulatory region of the *trp* operon of *Serratia marcescens*

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The nucleotide sequence of the regulatory region of the trp operon of Serratia marcescens is presented. It contains a transcription termination control site in the transcribed leader region preceding the first structural gene of the operon as well as a regulated promoter/operator transcription initiation region. The structural organisation of the leader region differs substantially from that of Escherichia coli.

In *Escherichia coli*, transcription of the *trp* operon is regulated at a promoter/operator¹⁻⁴, and also at an attenuator^{5,6}. The attenuator is a site within the transcribed leader region of the operon at which transcription is either terminated or allowed to proceed into the structural genes. Termination at this site seems to occur in response to the availability of charged tRNA^{Trp} for protein synthesis^{7,8}. RNA transcribed from the *trp* leader region contains a functional ribosome binding site⁹⁻¹¹, and may code for a short leader peptide with two adjacent tryptophan residues¹².

Comparison of the nucleotide sequences of the *trp* promoter/operator/leader region in *E. coli* and the related enterobacteria *Salmonella typhimurium* and *Shigella dysenteriae* have been useful in identifying the functionally important sequences within these regions¹²⁻¹⁴. The enterobacterium *Serratia marcescens* is more distantly related to *E. coli* and the sequence of its *trp* regulatory region would provide an interesting comparison. Although little is known of the structural organisation of the *trp* genes in *S. marcescens*, mutant and physiological studies indicate that many of these genes are arranged in an operon whose expression is coordinately regulated in response to changes in tryptophan concentration¹⁵⁻¹⁷. We have cloned a portion of the *S. marcescens trp* operon on plasmids and present here the nucleotide sequence of the regulatory region.

Cloning and restriction of *trp* operon

Chromosomal DNA of *S. marcescens* wild-type strain SM6 was cleaved with restriction endonuclease *SalI* and the fragments inserted into the *SalI* site of pBR322 (ref. 18). Plasmids carrying a functional *S. marcescens trpE* gene were isolated from the ligation reaction by selecting Trp⁺ transformants of an *E. coli* recipient strain in which most of *trpE* is deleted (see legend to Fig. 1). Each of several Trp⁺ transformants examined carried plasmids containing one or two inserts into the *SalI* site of pBR322. One typical representative, pGM14, was chosen for further characterisation. It carries a single 3,000-base pair insert; a restriction map of this fragment is shown in the top portion of Fig. 1. Although pGM14 confers prototrophy to *trpE*⁻ recipient strains, it does not complement lesions in any of the other *trp* structural genes, indicating that *trpE* is the only intact *S. marcescens trp* gene on pGM14.

S. marcescens trpE was located within the 3,000-base pair *SalI* fragment by constructing derivatives of pGM14 carrying portions of the insert. One such plasmid, pGM63, carries only the right-hand half of the 3,000-base pair insert, from the *SalI* site to the first *EcoRI* site (Fig. 1), but retains a functional *trpE*. This 1,500-base pair fragment could code for a protein of approximately 60,000 molecular weight, the estimated size²¹⁻²³ of *S. marcescens* anthranilate synthetase component I, the *trpE*

product. This suggests that the *EcoRI* and *SalI* sites delimiting the 1,500-base pair fragment must be very close to the ends of *trpE*. This was confirmed by DNA sequence analysis and comparison of the nucleotide sequences with the known N-terminal amino acid sequences of the *S. marcescens trpE* and *trpG* proteins²³. The DNA sequence at the *EcoRI* end of the fragment correlates precisely with the amino acid sequence at the beginning of the *trpE* protein, with the *EcoRI* site preceding the AUG start codon by 20 base pairs. There are two back-to-back potential AUG translation start codons at the beginning of *trpE* (Fig. 2). Using the first, the N-terminal amino acid sequences of the *trpE* proteins of *S. marcescens*, *E. coli* and *Sal. typhimurium* align perfectly²³. The *SalI* end of the fragment is located about 30 base pairs beyond the start of *trpG*, the structural gene for anthranilate synthetase component II, which must therefore follow *trpE* in the *trp* operon of *S. marcescens*^{16,26} (C.Y., G.F.M., G. Bennet and M. van Cleemput, in preparation). Thus, the 1,500-base pair *EcoRI*-*SalI* fragment carries *trpE* intact. However, the 20 base pairs preceding the start of *trpE* are insufficient to function as a promoter and we must therefore assume that in plasmid pGM63 *trpE* is expressed from a plasmid promoter. Most of the regulatory region of the *S. marcescens trp* operon is therefore absent from plasmid pGM63, but is contained in the parent plasmid pGM14. A detailed restriction map of the DNA region immediately preceding the *EcoRI* site is shown in the bottom portion of Fig. 1.

DNA sequence of the promoter/operator/leader region

A DNA segment of approximately 380 base pairs around the *EcoRI* site at the beginning of *trpE* was sequenced using several overlapping restriction fragments (bottom portion of Fig. 1). Large portions of the DNA sequence of the entire *EcoRI*₂₆₀ fragment and of the initial part of *trpE* (Fig. 2) are highly conserved between *S. marcescens*, *E. coli* and *Sal. typhimurium*^{3,12,13}. This suggests that the overall organisation of the regulatory region of the *trp* operon is similar in the three organisms. In particular, a DNA segment centred around base pair -10 of the *S. marcescens trp* operon is almost perfectly conserved. In *E. coli* and *Sal. typhimurium* this region is essential for *trp* promoter and operator function^{3,4,13,14}. Indeed, *E. coli* Trp repressor will protect the *HpaI* site at positions -9 to -14 and the *TacI* site (-20 to -23) but not the *HincII* site (-32 to -37) or the *HaeIII* site (+15 to +18) of *S. marcescens* from cleavage by the respective restriction enzymes (data not shown). As in the case of *E. coli* and *Sal. typhimurium*, the *S. marcescens trp* operon contains a leader region which separates the promoter/operator region from the start of the first structural gene, *trpE*. In *E. coli* and *Sal. typhimurium* this region is transcribed²⁷ and contains the *trp* attenuator^{6,12}.

In vitro transcription of the initial portion of the *S. marcescens trp* operon

When DNA fragments containing the *trp* promoter/operator/leader region of *E. coli* or *Sal. typhimurium* are transcribed with purified RNA polymerase *in vitro*, a major RNA transcript is produced as a result of transcription termination at the *trp* attenuator²⁸. To determine whether a similar termination site is

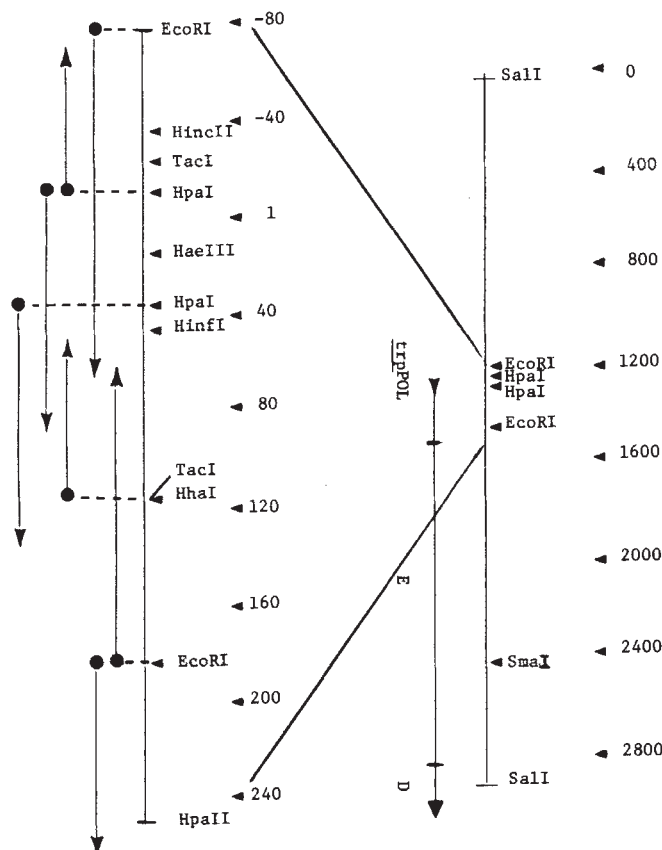


Fig. 1 Restriction map of a cloned fragment containing the initial portion of the *S. marcescens* *trp* operon. Chromosomal DNA of *S. marcescens* was prepared by the method of Miura and Sato¹⁹. 10 µg of chromosomal DNA and 5 µg of pBR322 DNA were digested with restriction endonuclease *SalI*, phenol extracted, ethanol precipitated and resuspended in 250 µl of ligation buffer (60 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP). The mixture was incubated in the presence of T4 DNA ligase for 2 h at 13 °C. Ligation was stopped by ethanol precipitation and the pellet resuspended in 100 µl TE-buffer (10 mM Tris-HCl pH 7.9, 0.1 mM EDTA). 20 µl were used to transform *E. coli* strain JA221 (C600 *r⁻m⁺ ΔtrpE5 leu recA*) to prototrophy. About 20 ampicillin-resistant, tetracycline-sensitive *Trp⁺* transformants were obtained. As *Trp⁺* transformants were readily obtained using several restriction enzymes and two plasmid vehicles we believe that the cloned *S. marcescens* fragments contain unchanged chromosomal sequences. The transformation frequency (10 transformants per µg DNA) was comparable to that reported for *E. coli* DNA²⁰. The restriction map of the insert carried by plasmid pGM14, a typical representative of the plasmids isolated from the *Trp⁺* transformants, is shown in the top portion of the figure. The arrow indicates the site of initiation and the direction of *trp* mRNA transcription. The bottom portion of the figure shows a detailed restriction map of the promoter/operator/leader region of the *S. marcescens* *trp* operon. Numbering starts at the presumed site of transcription initiation (position +1). The arrow indicates the direction and length of sequencing runs.

present in the leader region of the *S. marcescens* *trp* operon we isolated and transcribed various DNA fragments spanning the presumptive *trp* promoter/operator region. The transcripts obtained with the *EcoRI*₂₆₀ and the *HpaII*₄₀₀ fragments are shown in Fig. 3. The *EcoRI* fragment ends at position +185 in the leader region whereas the *HpaII* fragment extends to position +252 in the initial part of *trpE* (Fig. 1). Both fragments yield an approximately 175-nucleotide RNA transcript as the major product of *in vitro* transcription, together with a larger transcript of ~185 nucleotides for the *EcoRI* fragment and ~250 nucleotides for the *HpaII* fragment. As the 175-nucleotide transcript is also observed when larger DNA fragments or

plasmid pGM14 are used as DNA templates for *in vitro* transcription (results not shown), we conclude that the *S. marcescens* *trp* leader region contains a transcription termination site approximately 175 base pairs downstream from the transcription initiation site. Indeed, the DNA region between base pairs +150 and +180 shows close homology to the termination regions in the *E. coli* and *Sal. typhimurium* *trp* leader regions (Fig. 2)^{12,28}. The larger transcripts observed with both the *EcoRI*₂₆₀ and the *HpaII*₄₀₀ fragments (Fig. 3) presumably arise by readthrough beyond the transcription termination site to the end of the DNA fragments. Quantitative measurements indicate that there is about 30% readthrough at the *S. marcescens* *trp* leader termination site *in vitro*. In *E. coli* and *Sal. typhimurium* readthrough at the *trp* attenuator *in vitro* has been estimated at 5% and 30%, respectively²⁸. In experiments in which we have examined the function of the *S. marcescens* *trp* attenuator in *E. coli*, tryptophan starvation relieved transcription termination and the increase in operon expression apparently exceeded that typical of the *E. coli* *trp* operon. Hutchinson and Belser¹⁶ had previously noted that the regulatory response of *S. marcescens* to exogenous tryptophan was five times more sensitive than that of *E. coli*.

Features of the promoter/operator region

Both DNA-RNA hybridisation data²⁹ and protein primary structure comparisons^{23,30,31} indicate considerable evolutionary divergence in the *trp* operon of *S. marcescens* relative to those of *E. coli* and *Sal. typhimurium*. Conserved regions in such distantly related organisms may indicate functional importance. In particular, one might expect key sequences involved in recognition and binding of RNA polymerase and/or regulatory proteins to be conserved.

The promoter/operator regions of the *trp* operons of *E. coli* and *S. typhimurium* share a recognition sequence for *HpaI* (GTT↓AAC) (Fig. 2). The integrity of this 6-base pair sequence seems to be essential for both operator and promoter function, as a single base pair alteration within this region severely affects both functions¹⁴ (G. Zurawski, D. Elseviers and C.Y., unpublished). Of 19 base pairs in the symmetrical regions surrounding this *HpaI* site, 18 are conserved between *E. coli*, *Sal. typhimurium* and *S. marcescens* (Fig. 2). *E. coli* Trp repressor recognises this DNA segment in all three organisms. In *S. marcescens* it protects two restriction sites—the *HpaI* site between -9 and -14 and the *TacI* site between -20 and -23—from cleavage by the respective enzyme. The only difference between *S. marcescens* and *E. coli* in this region, a CG instead of a TA base pair at position -16, does not seem to affect repressor-operator recognition significantly, although it does reduce symmetry. Interestingly, an *E. coli* mutant with a different base pair change at the same position (TA→GC) is O^c (ref. 4), and Trp repressor does not protect its *HpaI* site against cleavage by *HpaI* (K. D. Brown and C. Y., unpublished results). The lack of sequence conservation from base pair -21 to -31 (Fig. 2) is consistent with the view that this DNA segment is not involved in repressor recognition⁴. The rightward endpoint of the region required for operator function in *E. coli* has been mapped to between positions -5 and +1 (ref. 4). The pattern of sequence conservation in this region (Fig. 2) suggests that residues to the right of position -3 may not be required for operator function. Taken together, these findings indicate that the *trp* operator of all three organisms is contained within the highly conserved DNA segment between residues -3 and -21.

RNA polymerase-promoter recognition and formation of a functional initiation complex are thought to involve sequence specific contacts with a DNA sequence around position -35 and with the 20-base pair DNA segment which immediately precedes the transcription initiation site^{13,32}. The pattern of sequence conservation between *E. coli*, *Sal. typhimurium* and *S. marcescens* is consistent with this notion. In addition to the highly conserved sequence between positions -3 and -21 the three organisms have an identical 6-base pair sequence between positions -32 and -37 (Fig. 2). This sequence, GTTGAC,

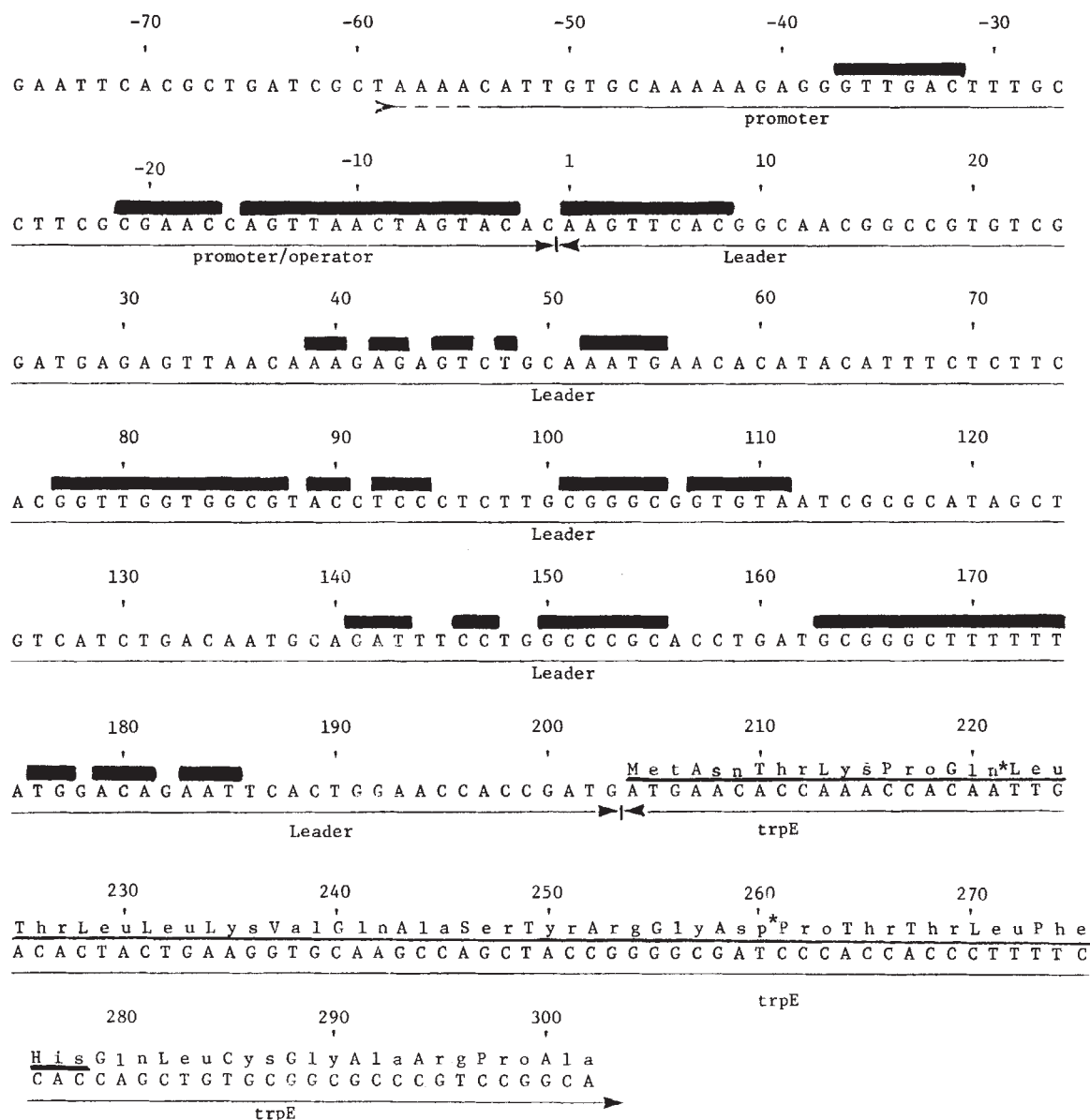


Fig. 2 Nucleotide sequence of the regulatory region of the *S. marcescens* *trp* operon. The sequenced segment comprises the promoter/operator/leader region and the initial portion of *trpE*. This subdivision is indicated by the arrows. Numbering starts at the presumed site of transcription initiation (residue +1). Clusters of base pairs that have been conserved between *E. coli*, *Sal. typhimurium* and *S. marcescens* are indicated by black bars. With two exceptions (Gln instead of His at residue no. 6 and Asp instead of Asn at residue no. 19) the underlined portion of the predicted amino acid sequence of *trpE* is identical to the previously published amino acid sequence at the N-terminus of *S. marcescens* anthranilate synthetase component I (ref. 23). The two differences are indicated by an asterisk. The DNA sequence predicts a recognition site for restriction endonuclease *Hae*III (GGCC) between positions 149 and 152. We find no cleavage by *Hae*III at this site. As the *Hae*III recognition sequence partially overlaps an *Eco*RII recognition sequence (CCTGG) between positions 146 and 150, we assume that methylation of the complementary C residue at position 149 prevents recognition by *Hae*III. DNA sequence analysis was carried out by the method of Maxam and Gilbert²⁴. Details of this and related procedures such as restriction site mapping, isolation of DNA fragments, phosphatase treatment, polynucleotide kinase reactions and agarose and polyacrylamide gel electrophoresis are given elsewhere^{3,25}.

recognised by *Hind*II and *Hinc*II, occurs at the same relative position in several unrelated promoters³³. The *S. marcescens* *trp* promoter is efficiently recognised by *E. coli* RNA polymerase both *in vivo* and *in vitro*. This suggests that the RNA polymerase of both organisms has similar requirements for its interaction with promoters. Initiation of transcription of the *S. marcescens* *trp* operon is likely to occur at the position previously determined for *E. coli*²⁷, particularly as 28 of 30 base pairs surrounding the potential initiation site (-21 to +9) are conserved. The importance of the highly conserved DNA segment between positions 1 and 8 following the transcription initiation site (Fig. 2) is unclear, as a deletion which replaces the DNA sequence beyond position +1 by a foreign sequence has no effect on either promoter or operator function in *E. coli*³.

Leader region

A 200-base pair leader region separates the site of transcription initiation of the *S. marcescens* *trp* operon from the start of the first structural gene *trpE*. There are many functional and structural similarities between this DNA segment and the leader regions of the *E. coli* and *Sal. typhimurium* *trp* operons¹² and the *E. coli* *phe* operon³⁴. They contain an attenuator region which terminates transcription in a run of TA base pairs preceded by a GC-rich segment. Although the residues at which transcription terminates have only been determined experimentally for the *E. coli* leader transcript^{35,36}, extensive sequence conservation in the region that contains the termination site in *E. coli* (Fig. 2; residues 140-180) suggests that in *S. marcescens* transcription termination must occur within the run of TA base pairs between

residues 169 and 175. This is consistent with the estimated length of 175 nucleotides for the terminated *S. marcescens* *trp* leader transcript synthesised *in vitro*.

Like the other *trp* leader transcripts, that from *S. marcescens* could generate considerable secondary structure. The structure depicted in Fig. 4 is remarkably similar to those proposed for the *trp* and *phe* leader transcripts of *E. coli* and the *trp* leader transcript of *Sal. typhimurium*^{28,34}. It has alternative stem and loop structures involving a GC-rich segment (residues 150–156) able to pair with two different regions of leader RNA. Base pairing with residues 162–168 generates the smaller stem and loop structure immediately preceding the 3'-OH end of the leader transcript. This structure has a calculated free energy of formation of $\Delta G \cong -17 \text{ kcal mol}^{-1}$ (refs 37, 38). Studies of termination relief mutants suggest that the integrity of the analogous structure in the *E. coli* *trp* leader transcript may be essential for efficient transcription termination³⁹. Residues 136–156 can also pair with residues 99–120 to form the alternative larger stem and loop structure, with a free energy of $\Delta G \cong -22 \text{ kcal mol}^{-1}$. With the exception of the sequences corresponding to the unpaired loop regions (residues 121–135 and 157–161), the entire DNA segment responsible for the formation of the alternative stem and loop structures (residues 100–170) is highly conserved between *S. marcescens*, *E. coli* and *Sal. typhimurium* (Fig. 2).

The *trp* and *phe* leader regions examined have in phase translation start and stop codons and may thus code for a short leader peptide. In *E. coli*, and AUG-centred ribosome binding site early in the *trp* leader region⁹ can function efficiently in initiation of translation both *in vivo* and *in vitro*^{10,11}. Two tandem tryptophan codons are found in the sequence coding for the putative *trp* leader peptides of *E. coli* and *Sal. typhimurium*¹², whereas seven phenylalanine residues are clustered in the C-terminal portion of the predicted *phe* leader peptide of *E. coli*³⁴. Two potential AUG translation start codons are present in the early part of *S. marcescens* *trp* leader RNA (Fig. 4). Both are in phase with the tandem tryptophan codons (residues 80–85) and a UAA translation stop codon (residues 110–112). The number of amino acid residues predicted for the *S. marcescens* *trp* leader peptide is either 19 or 28, depending on which initiation site is used (Fig. 5). Although in the other *trp* and the *phe* leader transcripts the translation stop codon immediately precedes the region involved in the formation of the alternative stem and loop structures, in *S. marcescens* the UAA stop codon is five amino acid codons beyond this point, within the region of assumed secondary structure (Fig. 4). A highly conserved sequence surrounds the tandem tryptophan codons at residues 80–85 (Fig. 5). The only nucleotide differences between *S. marcescens* and either *E. coli* or *Sal. typhimurium* in this region, U instead of C at position 88 and C in place of U at position 91, do not affect the predicted amino acid sequence. This conserved sequence is in the same position relative to the large stem and loop structure in all three *trp* operons. Although we believe that the extent of translation of the two tryptophan codons is an integral feature of the mechanism governing regulation of transcription termination⁴⁰, we cannot explain the extensive nucleotide and amino acid sequence conservation of the region surrounding the tryptophan codons.

Comparison of the initial portion of the *S. marcescens* *trp* leader region with the analogous regions in both *E. coli* and *Sal. typhimurium* reveals that the *S. marcescens* *trp* leader region contains a DNA insert of 27 base pairs between residues 9 and 10 of the *E. coli* and *Sal. typhimurium* sequences (Fig. 4). The 7 base pairs preceding this DNA segment (AGTTCAC; residues 2–8) are repeated with one difference at the end of the insert (AGTTAAC; residues 31–37). This extra DNA codes for the first of the two potential translation start codons (residues 26–28) for the *S. marcescens* *trp* leader peptide. The position of this AUG codon relative to the transcription initiation site is similar to that of the initiator codons for the *E. coli* and *Sal. typhimurium* leader peptides. The second potential start codon for the *S. marcescens* *trp* leader peptide is between residues 53

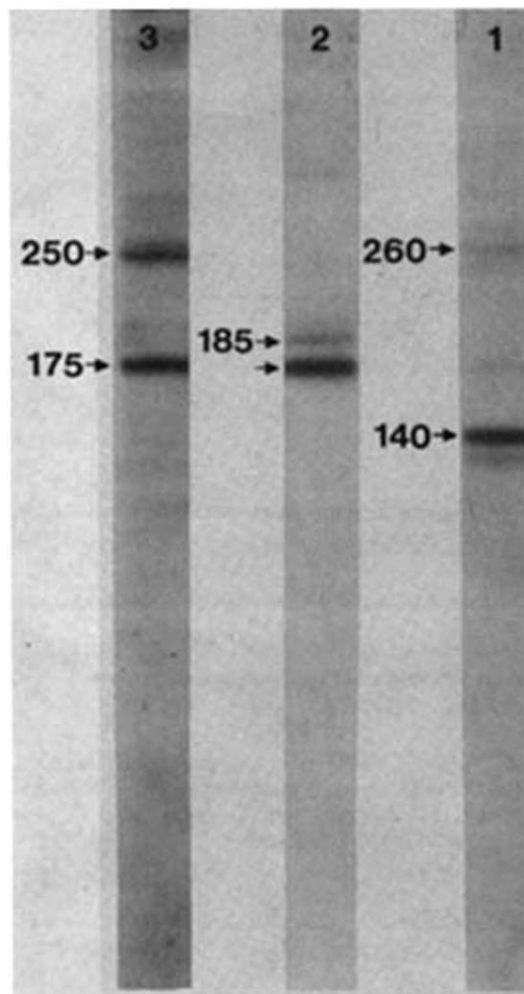


Fig. 3 *In vitro* transcription of DNA fragments containing *S. marcescens* *trpPOL*. RNA was transcribed from the following DNA fragments: (3) *S. marcescens* *HpaII*₄₀₀, (1) *S. marcescens* *EcoRI*₂₆₀, (1) *E. coli* *HpaII*₅₇₀. All fragments contain the regulatory region of the respective *trp* operons. The arrowed numbers indicate the length in nucleotides of the various transcripts. The size of the *S. marcescens* transcripts (slots 3 and 2) was calculated from the known lengths of the transcripts obtained with the *E. coli* *HpaII*₅₇₀ fragment²⁸. The 175-base RNA bands arise by transcription termination at the *S. marcescens* *trp* attenuator; the 185- and 250-base RNA bands are the result of transcription beyond the transcription termination site to the end of the respective fragments. The composition of the *in vitro* transcription mixture as well as the separation of the transcription products on polyacrylamide gels has been described²⁸.

and 55 (Fig. 5). The region preceding and including this AUG codon has been moderately conserved (Fig. 2). The region preceding either potential start site for the *S. marcescens* *trp* leader peptide shows no obvious homology to known ribosome binding sites or to the 3' end of 16S rRNA⁴¹.

We do not know whether either or both of the potential start sites is used in *S. marcescens*. Lee and C.Y.²⁸ have proposed a model for the regulation of transcription termination that is based on translation of the leader transcript and a shift between alternative RNA base pairing configurations in this transcript. Recent evidence suggests that base pairing between residues in the large loop of the alternative stem and loop structures and proximal segments of RNA may also be involved in attenuation. Thus, a stable hydrogen-bonded structure involving the large

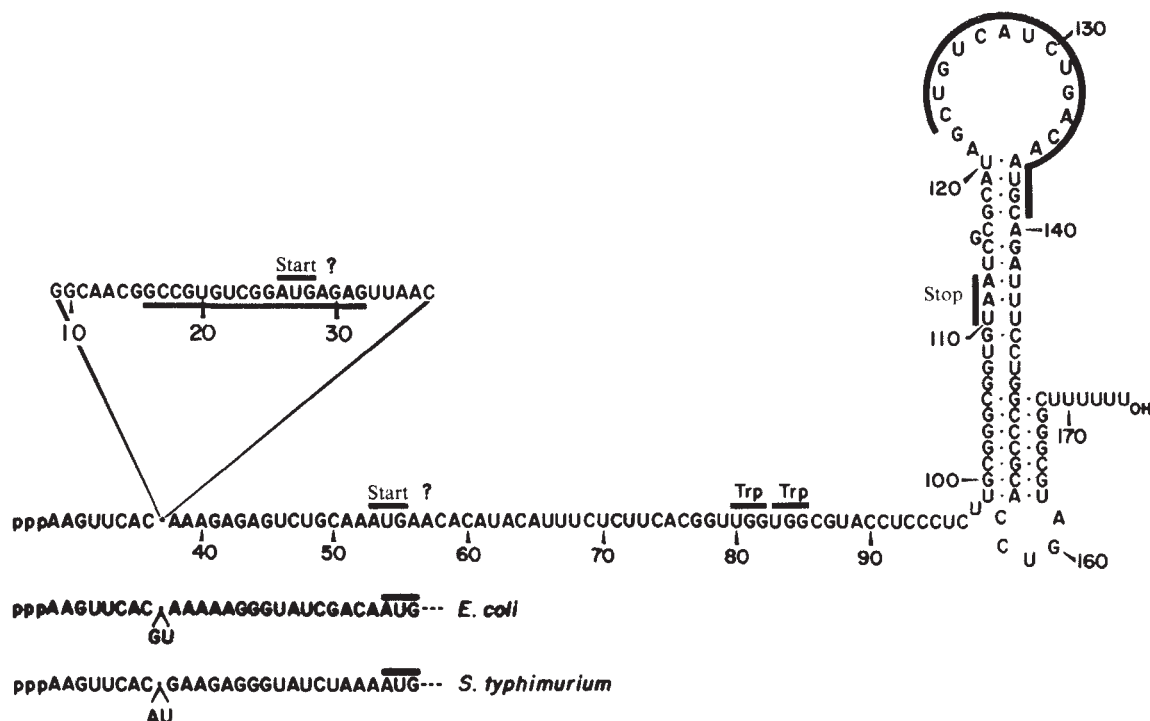


Fig. 4 Proposed secondary structure of the terminated *S. marcescens trp* leader transcript. The left hand portion of the diagram shows the position of the 27-base pair 'insert' compared to the sequence of the initial portion of the *E. coli* and *Sal. typhimurium trp* leader transcripts. The locations of the two possible translation start codons, the tandem tryptophan codons and the translation stop codon, are indicated. The regions of sequence homology between the insert and the large loop of the alternative secondary structures are indicated by underlining.

loop of the alternative stem and loop structures and the region containing the seven phenylalanine codons can be drawn for the *phe* operon transcript of *E. coli*³⁴. A similar, though weaker, complementarity between the region containing the two tryptophan codons and some of the residues of the large stem and loop exists in the *E. coli* and *Sal. typhimurium trp* leader transcripts⁴⁰. In *S. marcescens*, the complementarity (between residues 89–95 and 102–108) is restricted to the RNA region distal to the two tryptophan codons. However, a unique feature of the *S. marcescens trp* leader RNA is the strong complementarity between the region of the transcript which forms the large loop and the RNA sequence corresponding to the DNA insert (Fig. 4). Of 17 nucleotides between positions 16 and 32, 14, including the first AUG codon, are complementary to residues 139–123 of the *S. marcescens trp* leader transcript. The calculated free energy of formation of this structure is about $-10 \text{ kcal mol}^{-1}$.

Although many aspects of the functional and structural organisation are similar in all *trp* leader regions examined so far, three obvious differences exist between the *trp* leader region of

S. marcescens and the analogous regions of *E. coli* and *Sal. typhimurium*: (1) an extra segment of DNA, 27 base pairs long, inserted into the early portion of the *S. marcescens trp* leader region; (2) potential base-pairing between nucleotides within this insert and residues in the large loop of the alternative stem and loop structures; (3) the potential of *S. marcescens trp* leader RNA to code for a 19- or 28-residue leader peptide, with a stop codon within the region assumed to be involved in the formation of an alternative stem and loop structure. Despite these differences, the *S. marcescens trp* leader region terminates transcription both *in vitro* and *in vivo*, like the analogous regions in *E. coli* and *Sal. typhimurium*, and the extent of termination *in vivo* is a function of the availability of tryptophan. Our results suggest that regulation of transcription termination at the *trp* attenuator of *S. marcescens* may allow a greater range of expression than is attainable at the *E. coli trp* attenuator. The structural differences between the leader regions of *E. coli*, *Sal. typhimurium* and *S. marcescens* may therefore have developed to allow quantitatively different regulatory responses.

30	40	50	60	70	80	90	100	110
AUG AGA GUU AAC	AAA GAG AGU CUG CAA	AUG AAC	ACA UAC AUU	UCU CUU CAC	GGU UGG UGG	CGU ACC UCC	CUC UUG CGG GCG GUG UAA	
(Met-Arg-Val-Asn - Lys-Glu-Ser-Leu-Gln)	-Met-Asn -Thr-Tyr-Ile	-Ser-Leu-His	Gly-Trp-Trp-Arg-Thr-Ser	Leu-Leu-Arg-Ala-Val-stop				
<i>E. coli</i>	Met-Lys -Ala-Ile-Phe	-Val-Leu-Lys	Gly-Trp-Trp-Arg-Thr-Ser	stop				
<i>S. typhimurium</i>	Met-Ala -Ala-Thr-Phe	-Ala-Leu-His	Gly-Trp-Trp-Arg-Thr-Ser	stop				

Fig. 5 Predicted amino acid sequences of *trp* leader peptides. The predicted amino acid sequence of the putative *S. marcescens trp* leader peptide (second line) is compared with the predicted sequence of the analogous peptides of *E. coli* and *Sal. typhimurium*. The three peptides share an identical sequence of six amino acids surrounding the tandem tryptophan codons (indicated by a box). The *S. marcescens trp* leader peptide extends five amino acid residues beyond the end of the other peptides at its C-terminus. Depending on which of the two potential translation initiation sites is used, it may start at the same position as the *E. coli* and *Sal. typhimurium* peptides, or it may have nine additional amino acid residues at its N-terminus (indicated in parentheses).

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The regulatory region of the biotin operon in *Escherichia coli*

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It is proposed that the biotin anabolic operon in Escherichia coli is transcribed divergently from two partially overlapping face-to-face promoters. A mutation that increases transcription in vivo creates an additional promoter in vitro. The putative operator contains an imperfect palindromic sequence that partially overlaps the promoters. The regulatory and genetic implications of these findings are discussed.

THE synthesis of biotin in *E. coli* requires the products of the *bio* gene cluster^{1,2}, which is located at approximately 17 min on the genetic linkage map³. Transcription of the *bio* gene cluster is divergent^{4,5}. Transcription is initiated between the *bioA* and *bioB* genes and proceeds to the left into *bioA* and, from the opposite DNA strand, to the right into *bioB*, *F*, *C*, and *D*^{4,5}. Transcription in both directions is co-repressed by biotin^{4,6,7}. Genetic studies by Ketner and Campbell^{8,9} have shown that the *bioA* and *bioB* promoters are not independent, as several single mutations which increase *bioA* transcription decrease *bioB* transcription. The operators are not independent; a single mutation results in constitutive RNA synthesis in both directions⁹. In this report, we propose that the *bioA* and *bioB* promoters are situated face-to-face and partially overlap. The operator contains a hyphenated palindromic sequence that partially overlaps the promoters. This model is similar to one of the three models proposed by Ketner and Campbell⁹.

We have evidence that one of the mutations⁹ that increases leftward transcription, *bioP98*, may create a second leftward promoter. In addition, we report the nucleotide sequence of the *bio* regulatory region.

Identification and isolation of the *bio* regulatory region

Among the *bio* regulatory mutants isolated by Ketner and Campbell⁹ were promoter down mutants that were unable to

transcribe in either direction (Table I). The promoter down mutation, *bioP132*, was recovered by transduction with *λbio1* and the resultant phage, *λbio132*, was found to contain a DNA insertion element⁹, presumably within the *bio* regulatory region. (To simplify identification of bacteriophage *λbio1* derivatives, a shorthand description will be given to each transducing phage. This numbering⁹ does not infer isolation of an independent transducing phage with endpoints of the *bio* substitution different from *λbio1*. The phages described in this article are as follows: *λbio132* is *λbio1bioP98bioP132* and *λbio234* is *λbio1bioP98bioO34*. The DNA insertion, which seems to be IS1 from restriction site data, serves as a convenient physical marker for detecting restriction fragments that contain the *bio* regulatory region. Various restriction digests¹⁰ of *λbio1* and *λbio132* DNA were compared by gel electrophoresis^{11,12}. The *HindIII* sites in the *bio* substitutions of these phages are shown in Fig. 1. Further analysis resulted in the partial restriction map in Fig. 2.

It was necessary to locate the *bio* control region by another method as it is possible that the IS element could inactivate transcription by a mechanism unrelated to its insertion. Proof that fragments containing the site of the DNA insertion also contain the *bio* regulatory region came from the comparison of *in vitro* transcription of wild type (*λbio1*) and mutant (*λbio234*) DNA fragments. Transcription of the *HindIII* fragment from *λbio1* that was postulated to contain the control region (from the position of IS1 in an analogous fragment from *λbio132*) gave a major transcript of 170 nucleotides. This transcript terminated at the *HindIII* site and hybridised to the l-strand of *bio* transducing phage DNA, identifying it as a leftward (*bioA*) transcript.

On the other hand, a DNA fragment from *λbio234*, which contains the promoter up mutation, *bioP98*, and the operator constitutive mutation, *bioO34*, gave two major transcripts, one of 170 nucleotides and another of 150 nucleotides, both hybridising to the l-strand of *bio* transducing phage DNA. The longer transcript was initiated at the wild-type start site and the shorter transcript was initiated 24 nucleotides down from the wild-type

Table 1 Three classes of *bio* regulatory mutants

	Presence of <i>galT</i> and <i>E</i>	Phenotype		Properties
		Low biotin	High biotin	
Parent (<i>bio</i> Δ61)	+	Gal ⁻	Gal ⁻	Gal ⁻ at low biotin due to insufficient transcription.
I Promoter A up mutants P98 (spontaneous)	+	Gal ^S	Gal ^R	<i>bioA</i> mRNA increased 4- to 6-fold, <i>bioB</i> mRNA decreased to one-third wild type.
P2, P10, P43 (2AP)	-	Gal ^S	ND	
II Promoter A down mutants P130, P131, P132 (2AP)	+	Gal ⁻	Gal ⁻	No transcription in either direction, IS element in regulatory region.
	-	Gal ^R	Gal ^R	
III Operator constitutive mutants O34, O38, O49, O99 (spontaneous)	+	Gal ⁺	Gal ⁺	Constitutive in both directions, same amount of <i>bioA</i> mRNA as <i>bioP98</i> , <i>bioB</i> mRNA increased twofold.
	-	Gal ^S	Gal ^S	

The data in this table are summarised from ref. 9. The *bio* operons are under negative control with biotin as the co-repressor. Low levels of biotin (1.6 nM) permit synthesis of biotin, while high levels of biotin (41 nM) repress synthesis. Both a positive and negative selection technique were used to isolate control mutants. The parent strain contains a deletion (*bio*Δ61) which fuses *bioA* to *galE* and places the galactose operon under the control of the *bio* regulatory system. A portion of both *bioA* and *galE* are deleted in this strain. If *galK* is expressed, for example at low biotin concentrations, in the presence of galactose (glycerol is the carbon source) the accumulation of galactose-1-phosphate prevents growth. This is a negative selection for galactose utilisation. If, on the other hand, *galE* (and *galT*) is supplied by a transducing phage integrated at a distant site, galactose is metabolised and can serve as the sole carbon source. This is a positive selection for galactose utilisation. By using these two selections, Ketner and Campbell⁹ isolated the three classes of regulatory mutants listed. In each case, an arrow designates the ancestry of each of the mutant types. Thus the second and third classes of mutants all contain the *bioP98* mutation. Abbreviations are as follows: Gal⁻, unable to utilise galactose; Gal⁺, able to utilise galactose; Gal^S, sensitive to galactose; Gal^R, resistant to galactose; 2AP, induced with 2-aminopurine; ND, no data.

start site. These results correlate *in vitro* and *in vivo* transcriptional changes. If the shorter transcript were due to *bioP98* rather than *bioO34*, it would explain the total fourfold increase in leftward transcription in strains containing *bioP98* which does not change further on the formation of the double mutant, *bioP98*, *bioO34*. The *bioP98* mutation may increase transcription by creating a second leftward promoter, *bioP98*, whilst maintaining the wild-type *bioA* promoter. A precedent for overlapping promoters has been established in the galactose operon of *E. coli*¹³.

Early transcription experiments were carried out in the presence of heparin to produce selective transcription¹⁴. In these conditions, very little *bioB* transcription occurs. Later experiments were carried out in the absence of heparin, and transcription was initiated by the addition of magnesium chloride¹⁵. Selective transcription was maintained by using higher salt concentrations (see legend to Fig. 2). In this case, rightward transcription is increased to about one-half the level of leftward transcription.

Although we do not have direct genetic evidence for the positions of the *bioA* and *bioB* promoters, we have investigated the transcription of the *HinfI* fragment and the *HaeII* fragment that contain the control region (Fig. 2). These two fragments together encompass a region 340 nucleotides to the left and 500 nucleotides to the right of the *bioP98* promoter. In magnesium start conditions, two major transcripts could be found in each case; one leftward and one rightward. Transcripts from different fragments were shown to have the same start sites. Individual transcripts from the *HinfI* fragment were hybridised to the separated strands of the *HhaI* fragment containing the control region and the unhybridised portions of the RNA removed with RNase¹⁶. Fingerprints¹⁷ of the RNase-protected transcripts were essentially identical to those from transcripts from the *HhaI* fragment itself. Thus there are apparently only two major promoters in the 650-base pair *HinfI* fragment.

Organisation of the regulatory elements

Both DNA strands of the *bio* control region have been sequenced. The sequence between the *FnuEI* and *HphI* sites is presented in Fig. 3. There are several prominent features of the control sequences. First, there is a large imperfect palindrome near the *in vitro* mRNA start sites. The promoters are situated face-to-face and partially overlap. Both promoters overlap the palindrome, but the *bioB* promoter does so to a much greater extent. The promoter sites are A-T rich (46/65 base pairs) and are flanked by relatively G-C rich sequences (16/25 and 9/19 base pairs). The first AUG codons of the two mRNAs are 86 nucleotides apart on the DNA sequence. It is not yet known whether these represent the initiation sites for the synthesis of the *bioA* and *bioB* gene products.

Positions of the promoters

There are three possible positions for the two *bio* promoters: (1) overlapping; (2) face-to-face and non-overlapping; (3) back-to-back and non-overlapping⁹. The first model can be divided into a spectrum of models that range from overlapping face-to-face to overlapping back-to-back promoters. The proposed promoters are found to conform to the first model with face-to-face

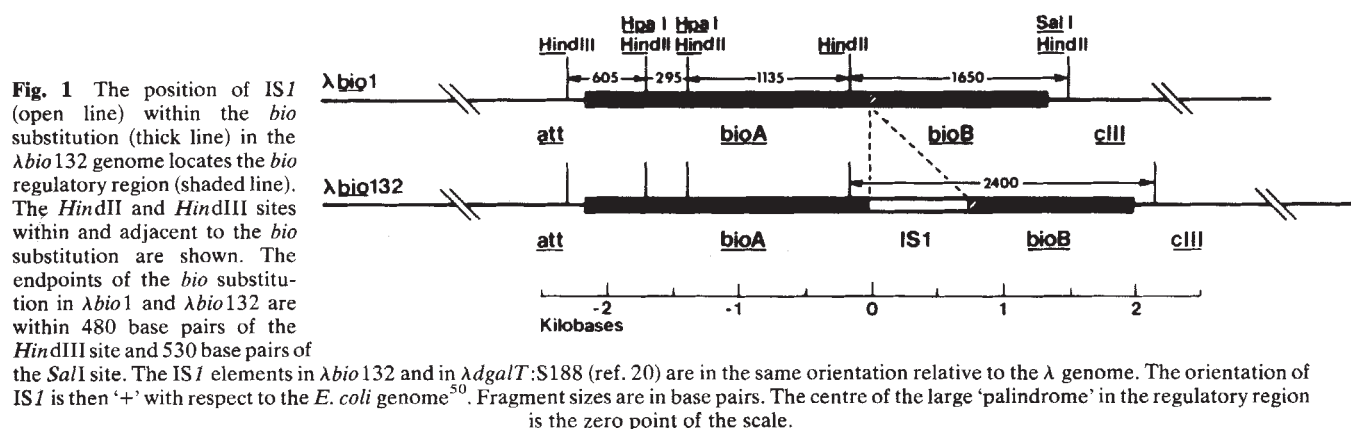
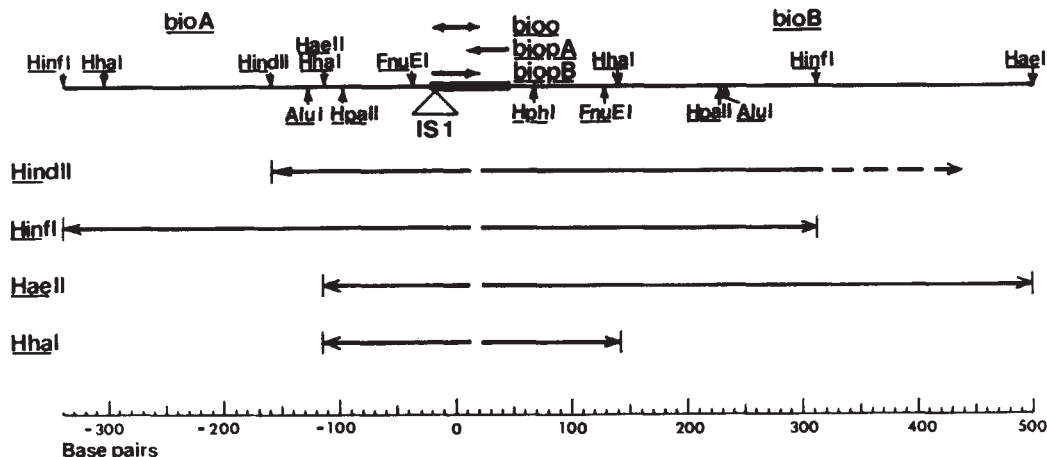


Fig. 1 The position of IS1 (open line) within the *bio* substitution (thick line) in the λ bio132 genome locates the *bio* regulatory region (shaded line). The *HindIII* and *HindIII* sites within and adjacent to the *bio* substitution are shown. The endpoints of the *bio* substitution in λ bio1 and λ bio132 are within 480 base pairs of the *HindIII* site and 530 base pairs of the *SalI* site. The IS1 elements in λ bio132 and in λ dgatT:S188 (ref. 20) are in the same orientation relative to the λ genome. The orientation of IS1 is then '+' with respect to the *E. coli* genome⁵⁰. Fragment sizes are in base pairs. The centre of the large 'palindrome' in the regulatory region is the zero point of the scale.

Fig. 2 The first restriction sites flanking the *bio* regulatory region are shown for several sequence-specific endonucleases. The thick line represents the region containing the *bioA* and *bioB* promoters. The positions of the promoters (*bioPA* and *bioPB*) and the operator (*bioO*) are shown by arrows above the linear map (see Fig. 3). The approximate position of the IS1 insertion in *bioP132* is shown. The lower portion of the figure shows the transcripts, (arrows), obtained from



fragments listed at the left. Only two initiation sites were observed in the entire 840 nucleotide region that is cut from the 1,650-base pair *Hind*II fragment, represented as a broken line, terminates prematurely at several sites. Reactions were carried out in a final volume of 20 μ l with approximately 0.05–0.2 μ g of a purified DNA fragment (about 0.05, 0.1, 0.2, 0.5, 1.0, 100 mM KCl and 10 mM 2-mercaptoethanol. RNA polymerase holoenzyme, 8.6 μ g (840 nM or an RNA fragment, 0.05–0.2 μ g was added to the reaction mix and incubated at 37°C for 5 min. The reaction mixture was then transferred to a reaction mixture containing ribonucleoside triphosphate at a final concentration of 10 μ M for pyrimidine nucleoside triphosphates and 200 μ M for purine nucleoside triphosphates, as well as the remaining required ribonucleoside triphosphates at final concentrations of 200 μ M. The reaction was carried out for 5 min at 37°C and the reaction started by adding $MgCl_2$ to a final concentration of 10 mM. After 20 min of RNA synthesis, the reaction was worked up as described previously¹⁴.

partially overlapping promoters. The *in vitro* *bioA* and *bioB* mRNA start sites are 10 nucleotides apart on the DNA and the messengers do not overlap. This observation rules out the second model, of two distant face-to-face promoters with overlapping transcripts, which was not ruled out genetically. The third model of two independent and distant back-to-back promoters has been proposed by Das Gupta *et al.*¹⁸ on the basis of electronmicrographic evidence, but it is not supported by the promoter interactions found in the genetic studies⁹. However, the genetic argument requires that the promoters are functionally independent, which is not substantiated in the singular case of the interactive distant back-to-back promoters of the arabinose operons of *E. coli*¹⁹. Das Gupta and his collaborators believe that the mRNA start sites are about 174 nucleotides apart¹⁸ but we have not found transcripts separated by this distance in our studies. We must assert, however, that only the position of the *bioP98* promoter can be uniquely and unambiguously determined by mutation. The *bioA* and *bioB* promoters are positioned solely by the fact that *in vitro* transcription starts at two positions within an 840-base pair region that contains portions of the *bioA* and *bioB* genes.

The proposed orientation of the promoters is consistent with the genetic data. The *bioP98* mutation, which increases total leftward transcription fourfold, decreases rightward transcription to one-third of the wild-type level⁹. Because the promoters overlap, mutations in one promoter may affect the other promoters. It is also possible that operator mutants affect promotion, and promoter mutants may affect repression, as the putative operator partially overlaps the promoters. The binding of RNA polymerase to the *bioA* or *bioP98* promoters could even sterically prevent binding of RNA polymerase to the *bioB* promoter.

A second genetic observation that is explained by the proposed model has been noted by Ketner and Campbell⁹. The insertion sequence element found in the *bio* regulatory region could inactivate rightward transcription by insertion into the *bioB* promoter and could block transcription from the *bioA* promoter by termination within the *IS1* element. *IS1* is known to give polar mutations when inserted in either direction²⁰. In this regard, we have mapped the insertion site to a region near or in the *bioB* promoter and to the left of the *bioA* *in vitro* mRNA start site (Fig. 2).

Finally, the proposed promoter orientation may explain an observation of Das Gupta *et al.*¹⁸. They observed transcriptional intermediates using the electron microscope in which one DNA strand had been displaced by an RNA strand synthesised in either direction, but not in both directions. For those intermediates observed, the binding of a second RNA polymerase molecule is precluded because the second promoter is in the form of an RNA-DNA hybrid.

Location of the operator

The selection of operator mutants that are simultaneously constitutive for transcription in both directions suggests that the promoters for both operons must share at least one common operator site⁹. However, studies of the repression of the *bio* operons with biotin analogues suggest the possibility of two independent operators²¹. Preliminary sequence analysis of the double mutant, *bioP98*, *bioO34*, shows two sequence changes. One of these is at position -3 (Fig. 3) and the other is at position +15. They are both located within the imperfect palindrome. We conclude that the imperfect palindrome is at least one component of the operator, but as we have sequenced neither mutation alone we cannot assign each mutation an identity. Because the *bio* repressor has not been isolated, it is not possible to locate the operator by binding studies; however, it is possible to select plasmids containing the *bio* operator by their ability to titrate repressor (A. O. and J. A., unpublished). The *bio* repressor(s), like the *gal*, *trp*, *lac* and λ phage repressors (reviewed in ref. 22), may effect repression by isosterically preventing RNA polymerase binding rather than by allosteric interaction.

Promoter sequences

It is difficult to define the exact molecular interactions between RNA polymerase and a given promoter. The physical limits of promoters have been approximately defined by mutations and binding or protection studies^{13,23-43,49}. However, most sequenced promoters have two common interaction sites. These are the Pribnow box²⁵ and the recognition site²⁷, both defined by homologies found in sequenced promoters, as well as, to a certain extent, by mutations or protection studies (reviewed in

refs 23,24). The Pribnow box is of the form 5'TATRRTR3' (R, a purine nucleotide) and is centred at approximately 10 nucleotides proximal to the RNA initiation site. The A in the second position and the T in the sixth position from the 5' end of the Pribnow box are the most highly conserved bases¹³. In the *bioA* and *bioB* promoters four out of seven bases match the Pribnow paradigm (Fig. 4). There is an A residue in the second position and a T residue in the sixth position of the Pribnow box in each promoter. In the case of the *bioP98* promoter, four out of seven bases agree with the Pribnow sequence. The tentative C to A substitution in the *bioP98* promoter allows agreement with the second nucleotide of the formal Pribnow sequence. Because both the sequenced operator and promoter mutations occur within the *bioP98* promoter, the importance of the substitution at position -3 awaits sequencing of the *bioP98* mutation alone.

The second common feature of most promoters is a recognition site which encompasses about 12 nucleotides centred at approximately position 32 upstream of the RNA initiation site. There are two general types of recognition sites. However, there are some promoters that do not fit into either class very well, for example the *lacI* promoter⁴², and others that are between classes, for example the *araB* promoter⁴³. Nevertheless, it is convenient to divide the promoters into groups with recognition sequences most closely resembling the sequences TGTGACAATTT, referred to here as type I promoters²³⁻³⁵, and those resembling the sequence NNNNNACACTTT (N is any nucleotide), referred to here as type II promoters^{13,23,36-41}. The former type is quite often found in bacteriophage promoters²⁵⁻³³ and the latter in the catabolite activator protein

(CAP) regulated sugar utilisation operons^{13,23,36-40} in *E. coli*. The *bioA* promoter contains a recognition site that agrees with 9 of the 12 bases in the type I recognition site. However, there are only 5 bases between the *bioA* recognition site and the Pribnow box, in contrast to a distance of 12 to 14 bases for most promoters. Perhaps this distance can be reconciled by the possibility that two different subunits of RNA polymerase act at the two sites. Alternatively, the true recognition site may reside further from the Pribnow box and contain less homology to known recognition sites. The recognition site of the *bioB* promoter matches the type I sequence in five positions. This is not an unusually poor fit as several type I promoters agree with the type I sequence at only six positions^{27,33}. The *bioP98* promoter fits at seven positions in the type I recognition sequence. The average match for the type I promoters evaluated here²⁵⁻³⁵ is 8 out of 12 positions.

It is difficult to explain the relationship of the *in vivo* transcriptional levels to the primary sequence of the promoters. The *bioA* promoter produces one-twentieth of the molar amount of mRNA, when fully de-repressed in a *Bio*⁻ strain, compared to the fully induced *galE* promoter⁹. The *bioA* and *bioB* promoters transcribe at approximately equal efficiencies *in vivo*⁴. The *bioP98* promoter is three times more efficient *in vivo* than the wild-type *bioA* promoter⁹. However, there is no obvious correlation between these promoter sequences and the resulting transcriptional levels. The *bioA* and *bioB* promoter sequences are quite different, yet they produce RNA equally well. It is not clear what features cause these promoters to be twenty times less efficient than the *galE* promoter. Lastly, because the *bioP98* and

Fig. 3 The overlapping face-to-face *bioA* and *bioB* promoters are positioned on the DNA sequence by the 5' ends of the *in vitro* mRNAs as well as homology to other promoters²³⁻⁴³. The *bioP98* promoter, created by mutation, is positioned by the 5' end of the mRNA as well as the tentatively assigned base substitutions at positions -3 and +15 found in the double mutant, *bioP98*, *bioO34*. The promoters are defined to be DNA sequences starting from and extending 35 to 40 nucleotides upstream from the initiation site for transcription. This definition is based on studies of DNA regions essential to promoter activity²³. The *bioB* promoter and the *bioP98* promoter overlap the putative operator to a greater extent than the *bioA* promoter (lines above and below the DNA sequence delineate the imperfect palindrome). Transcription may be repressed sterically either by binding of repressor to the operator or by steric competition of RNA polymerase molecules for one of the several overlapping promoters. Neither the presumptive sequence change in the *bioO34* mutation, which abolishes repression *in vivo*, nor the presumptive *bioP98* sequence change, which increases total leftward transcription fourfold, affects sequences in the recognition sites (open lines) or Pribnow box structures²³ (shaded lines) of the *bioA* or *bioB* promoters. Both strands of the DNA have been sequenced from positions -21 to 53 by the method of Maxam and Gilbert⁵¹. RNA sequence data from the *bio* transcripts will be presented elsewhere. Sequences that are complementary to the 3' end of 16S rRNA⁵² are underlined and the first AUG sequences in each RNA are enclosed in boxes.

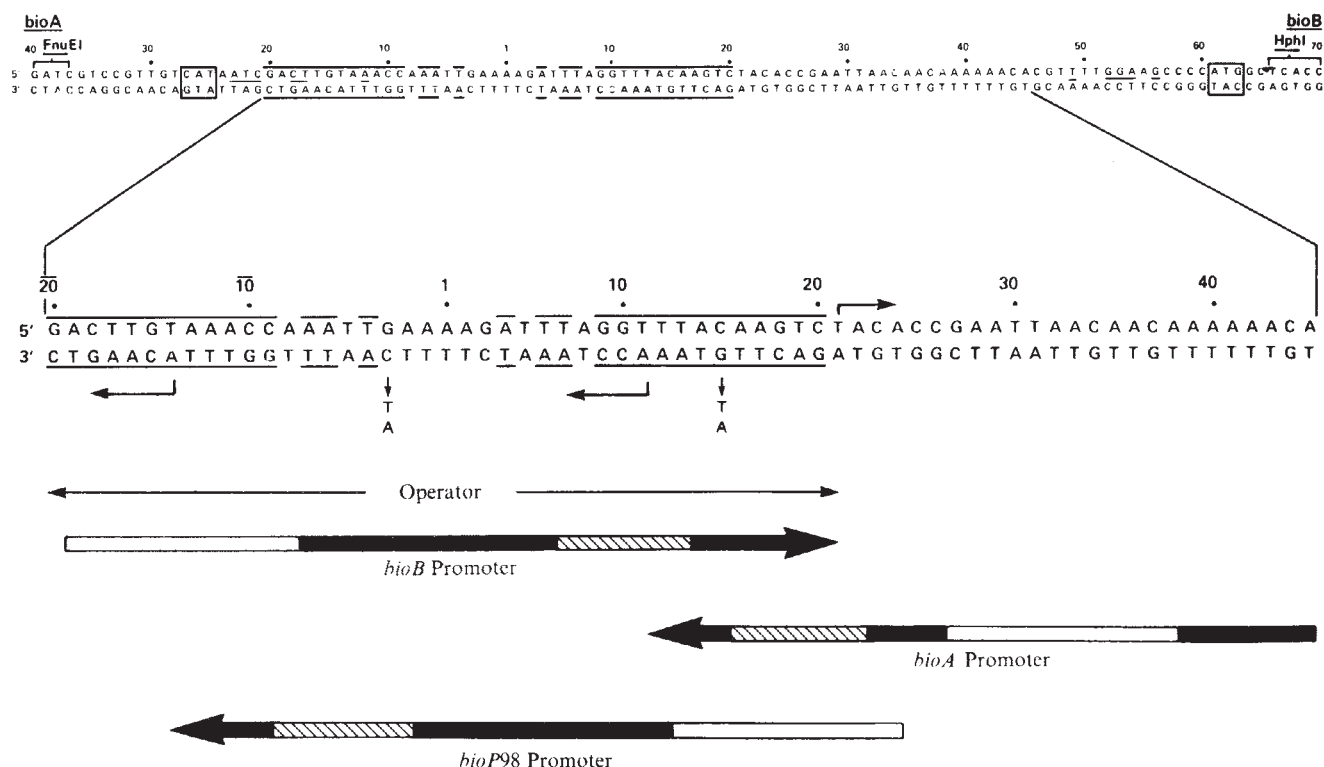


Fig. 4 The *bio* promoters are compared to the tryptophan³⁵ and lactose³⁶ operon promoters of *E. coli*. All promoters are aligned by their Pribnow boxes. The wild-type *bio* promoters are dissimilar in sequence, though the *bioP98* and *bioB* promoters share extensive homology due to the imperfect palindrome. All the *bio* promoters are more closely related to the tryptophan operon or bacteriophage promoters than to the lactose operon promoter. Sequences that are similar to the recognition site of the *trpE* promoter are enclosed in boxes. In the *bioA* promoter, these 'recognition' sequences are unusually close to the Pribnow box. The base substitutions in the *bioP98* promoter are underlined. The mRNA starts (arrows) were determined by an indirect method⁵³. RNA samples were divided into two aliquots and one aliquot was treated with bacterial alkaline phosphatase to remove the triphosphate at the 5' end. Comparison of RNase T1 fingerprints of the two samples immediately identified the 5'-oligonucleotide. We cannot yet rule out initiations of leftward transcription at positions 8, 9 or 10 or of rightward transcription at position

<i>bioA</i>	A A A C G T G T T T T T T G T T G T T A A T T C G G T G T A G A C T T G T A A A C C T A A A T
<i>bioB</i>	T C G A C T T G T A A A C C A A A T T G A A A A G A T T T A G G T T T A C A A G T C T A C A C
<i>bioP98</i>	C G G T G T A G A C T T A T A A A C C T A A A T C T T T T A A A T T T G G T T T A C A A G T C
<i>lacZ</i>	A G G C T T T A C A C T T T A T G C T T C C G G C T C G T A T G T T G T G T G G A A T T G T G
<i>trpE</i>	A G C T G T T G A C A A T T A A T C A T C G A A C T A G T T A A C T A G T A C G C A A G T T C

20. It is unusual that the rightward transcript is initiated with uridine-5'-triphosphate.

bioB promoters overlap the 'palindrome,' they share considerable homology. However, these promoters are not equally utilised *in vivo*.

Operator sequences

The putative *bio* operator, like the *lac*⁴⁴, *trp*³⁵, *gal*⁴⁰ and phage λ (ref. 30) operators, is an imperfect palindromic sequence and suggests a symmetrical repressor interaction. The imperfect palindrome is larger than any other sequenced operator and bears no sequence resemblance to any of the other operators. The palindrome could be increased by deletion of the A-T base pair at position 8 or an insertion of an A-T base pair between positions -8 and -9.

Comparison with other control regions

Recently, considerable progress has been made in determining the DNA sequences of several regulatory regions²³⁻⁴³. Comparative studies of the overall organisation of control regions as well as the individual regulatory elements have become possible. The *bio* system incorporates some of the general features of the unidirectional operons. Like *gal*, *lac*, *trp* and λp_L , the 'palindromic' operator overlaps the promoters. In the *bio* system we find an unusual transcriptional pattern; two transcriptional units are coordinately regulated from a single control region and both code for enzymatic activities rather than there being a single operon for a regulatory factor and another for enzymatic activities. Other examples of divergent transcription are the *argECBH* cluster⁴⁵, the $\lambda p_R - p_{rm}$ system⁴⁶ and the *araCBAD* locus⁴⁷.

In both $\lambda p_R - p_{rm}$ and *ara*, the rationale for divergent transcription is to permit the modulation of autoregulation. In the $\lambda p_R - p_{rm}$ region, three operators overlap both back-to-back and overlapping promoters⁴⁶. A low level of repressor both stimulates leftward transcription from p_{rm} to produce more repressor protein and decreases rightward transcription from p_R (ref. 14). As repressor accumulates, all the operator sites become occupied and transcription from p_{rm} is shut off. In *ara*, the *araC* gene product acts both as a repressor of its own synthesis and an activator of the arabinose catabolic operon in the presence of arabinose^{47,48}. Again, the two operons are interactive, transcription of *araC* is not independent of transcription of *araBAD*¹⁹. Thus, in these operons, divergent transcription permits fine tuning of the primary metabolic operon and the operon coding for the autoregulated control protein. This is not the case for the *bio* cluster as none of the *bio* genes are required for the expression of remaining *bio* genes⁹.

In summary, the *bio* regulatory region has been located by two independent methods. It is proposed that the *bioA* and *bioB* promoters are located face-to-face and partially overlap. Direct evidence that the promoters are in the proposed positions must

come from sequence studies of individual promoter mutations or from the 5' end sequences of the *in vivo* mRNAs because all *in vitro* binding or transcription assays are potentially subject to artefacts. It nevertheless seems unlikely that the same small DNA region that contains the site where insertion of IS1 inactivates *in vivo* transcription of both *bioA* and *bioB*, and that possibly contains the *bioP98* and *bioO34* mutations, should coincidentally contain the sites for the initiation of *in vitro* transcription. The proposed operator site contains or is entirely composed of an imperfect palindromic sequence. A new promoter may be created by the *bioP98* mutation.

Detailed descriptions of the RNA and DNA sequence determinations from positions -180 to +150 will be presented elsewhere.

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letters

Preferred frequency for interstellar communications

MANY scientists believe that intelligent life is common in our Galaxy¹⁻⁶ and that electromagnetic waves are the most practical means of communication^{7,8}. Even equipment of a terrestrial standard can produce communication within a distance of 100 light yr. Several searches for interstellar signals have been proposed; however, difficulty arises in the selection of technical parameters such as frequency and coding. Among these, the selection of frequency is the most important. We propose here that the 4,829.659-MHz line frequency of the $1_{11} \rightarrow 1_{10}$ rotational transition of formaldehyde is a plausible frequency.

Simple calculations show that the number of possible frequency channels is so large that a search is not realistic, and we can assume that another party wishing to communicate

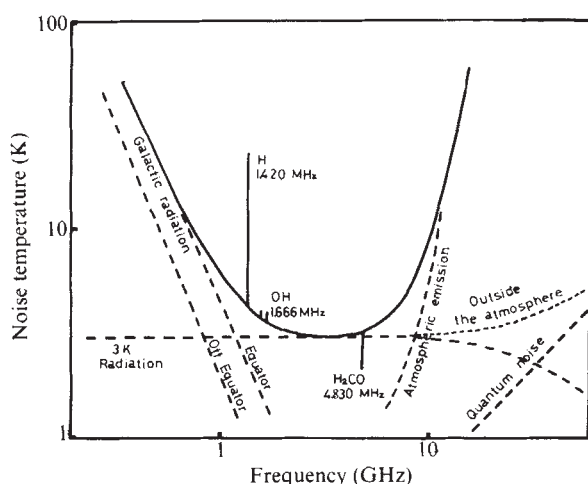


Fig. 1 The background noise temperature plotted against frequency. The solid line shows the total noise temperature on the surface of the Earth. Outside the atmosphere, the influence of atmosphere can be neglected in high frequencies and the total noise temperature is shown by the short-dashed line. The long-dashed lines show each component which sums up to form total noise emission. The absorption line at 4.83 GHz is the formaldehyde absorption band in the direction of dark clouds.

Table 1 Candidate stars in the direction of Coal Sack Nebula taken from the Michigan Spectral Catalogue

No.	HD no.	Spectral type	Photographic magnitude	Position (1900.0)	
				RA	dec
1	109478	F0	9.2	12h 29.8min	-64°43'
2	109622	G5	9.9	12 30.9	-63 25
3	109699	K4/M0	9.6	12 31.5	-62 10
4	109811	F0	9.7	12 32.5	-64 47
5	109819	F8/G0	9.3	12 32.6	-62 22
6	110040	K5	10.3	12 34.2	-61 11
7	110310	F0/2	7.9	12 36.0	-64 11
8	110735	F6/7	9.0	12 39.0	-61 40
9	110830	F2/3	9.1	12 39.7	-61 38
10	111002	G0/2	9.9	12 41.0	-60 52
11	111455	G5/8	9.8	12 44.2	-61 54
12	111557	F3/5	9.3	12 44.9	-62 27
13	111580	F5	9.1	12 45.1	-64 22
14	111778	K0	10.0	12 46.5	-61 50
15	111905	F7/G0	10.0	12 47.4	-63 34
16	112109	F0	8.2	12 48.9	-63 6
17	112169	F2	9.3	12 49.4	-62 35
18	112216	G0/2	9.5	12 49.8	-62 4
19	112513	M2	10.2	12 52.1	-62 17
20	112636	G3	9.0	12 53.0	-62 23
21	112703	F5	8.0	12 53.5	-63 50
22	112752	K5	9.9	12 53.8	-64 32
23	112795	F3/6	9.7	12 54.1	-63 41
24	112854	G8	9.5	12 54.5	-62 7
25	113015	G0	10.0	12 55.6	-61 56
26	113152	F2	9.1	12 56.5	-60 47
27	113181	K5	11.2	12 56.7	-62 38
28	113182	K0	9.7	12 56.7	-62 56
29	113375	K3	9.5	12 58.1	-64 15
30	113466	G5	9.5	12 58.8	-63 42
31	113707	G3	10.3	13 0.4	-61 25
32	113824	F5	10.5	13 1.2	-61 4
33	113844	F8	10.0	13 1.3	-61 12
34	113860	F0	9.4	13 1.4	-63 28
35	114091	K5	10.2	13 3.1	-64 11
36	114252	F0	9.7	13 4.2	-61 18

would also consider this unpractical. Thus we need to predict the other party's decisions, and we can rely only on the common knowledge between the two parties. This also applies to the other party, so certain discrete frequency channels can be specified.

Cocconi and Morrison⁷ suggested the use of neutral hydrogen line frequency of 1,420.406 MHz which was then the only known radio spectral line in the Galaxy. If transmitted at this frequency, the possibility that the signal could be detected by chance by some unknown party is higher than at other frequencies: it would help to establish 'implicit mutual agreement'.

The 4,829.659-MHz H_2CO line, because of the anti-maser effect, exhibits a very low excitation temperature⁹. Due to this anti-maser absorption, the background radiation temperature in the directions of dark clouds is below the 3 K background temperature. If a search is made at this frequency, we get a lower noise temperature than at any other frequency bands (the opposite is true for the hydrogen 21 cm line). Although the background noise temperature is lowered only by ~ 1 K, the depression itself could be interpreted as a significant marker.

A civilisation on a star which we see in front of a dark cloud must be aware of this advantage and would transmit signals at this frequency knowing that we know of the same advantage and could forecast their decision. One can specify the frequency of this absorption minimum within 5 kHz (300 m s^{-1} in Doppler shift) due to low temperature dark clouds. The absorption line is narrower than other lines, but above all, the frequency of the transmission signal can be set to the absorption minimum determined by observations and the search band is much narrower compared with other proposed bands. Properties of this frequency band as discussed above meet the requirements of the beacon signal necessary to establish the first contact. If intelligent civilisations are common throughout the Galaxy, they must have formed a community using communication and must be sending beacon signals.

There are many dark clouds, covering a substantial fraction of the sky and many stars that can be seen in front of such clouds. A characteristic of our method is that we can specify not only the frequency but also the directions to be searched.

Candidate stars in the direction of the nearest dark cloud, Coal Sack Nebula, are listed in Table 1. These are the stars of luminosity classes IV and V with the spectral types F, G, K and M in the Michigan Spectral Catalogue¹⁰.

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The controversial carbon solid-liquid-vapour triple point

THE controversy over the solid-liquid-vapour (S-L-V) triple point of carbon began in 1849 (ref. 1). Since then, investigators have developed widely differing points of view which are discussed here. Some believe the triple-point pressure is $\sim 10^5$ Pa (~ 1 atm), whereas others believe it is $\sim 10^7$ Pa (~ 100 atm). Over the past 40 yr, the 10^7 Pa view has dominated but the evidence is not entirely convincing. In the past few years, new evidence has been obtained that apparently ends the controversy at least from the standpoint of the proper order of magnitude of the pressure.

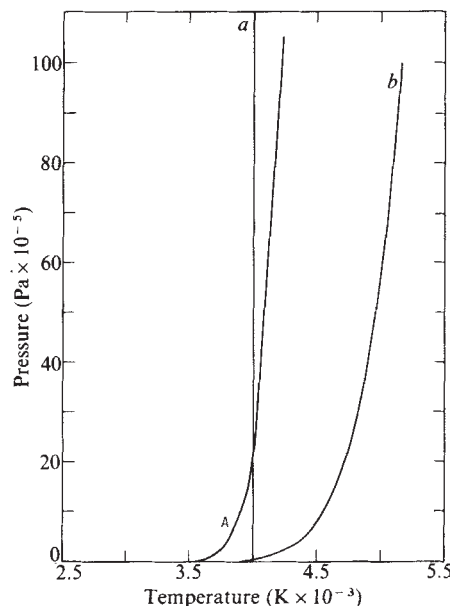


Fig. 1 Solid-liquid and solid-vapour boundaries for the low-pressure region of the carbon phase diagram. a, Bundy; b, JANAF.

In any single-component phase diagram, the S-L-V triple point is located at the intersection of the solid-liquid (S-L) and solid-vapour (S-V) boundaries. These boundaries for carbon are shown in Fig. 1. The S-L boundary was obtained by an extrapolation of Bundy's high-pressure data, and the JANAF data gives the S-V boundary^{2,3}. Even though Bundy's extrapolated data could be in error by 100° or 200° , these boundaries (Fig. 1) indicate that the S-L-V triple-point pressure must be of the order of 10^5 Pa. However, the S-L-V triple point was believed to occur at $\sim 10^7$ Pa and 4,200 K, and it was considered that the JANAF calculations above $\sim 3,000$ K might be wrong. Thus, S-V boundaries, such as A in Fig. 1, were proposed, even though there was little experimental basis⁴. The question of which S-V boundary is correct can be answered by considering the results of several studies on the vapour pressure of carbon in the region of 4,000 K.

Covington, Liu and Lincoln⁵ and Lundell and Dickey⁶ have obtained vapour pressure data that confirmed the JANAF data in the region of 10^5 – 10^7 Pa. Also, Maurer *et al.*⁷ obtained data at $\sim 10^4$ Pa that agreed with the JANAF curve. Data obtained several years ago indicated that graphite transforms to the more stable carbyne forms of carbon above $\sim 2,600$ K. Hence, the equilibrium S-V boundary must lie below the JANAF curve. All these data are shown in Fig. 2 in the region of the S-L-V triple point. I have recently reported¹¹ preliminary data on the rate of transformation of graphite into each of two of the carbyne forms¹¹. These data also show that the lowest transformation temperature is $\sim 2,600$ K and supports the conclusion that the equilibrium vapour pressure of carbon must be less than the JANAF values above this temperature. These rate data show that the transformation graphite $\rightarrow$ carbyne is relatively slow up to 2,800 K (first-order rate constant of $\sim 10^{-5}$ s at 2,800 K). There is some evidence that the transformation rates increase with temperature as expected, but 2 or 3 s are required for 80–90% conversion in the region of 3,800 K. Therefore, the transformation did not have time to take place in the experiments of Covington, Liu and Lincoln because their measurement time was of the order of 1 ms or less. Hence, their data would be expected to fall on the JANAF curve because it represents the vapour pressure that graphite would have if it were the stable form above $\sim 2,600$ K. In the experiments of Lundell and Dickey, it is difficult to estimate the time required to heat and vaporise the carbon because the beam traversed through the sample at an appreciable rate. However, it seems

that the time available for transformation of the carbon was well below 1 s, especially at the higher power densities. Therefore, their data would be expected to fall on the JANAF curve. These five groups of data constitute a mutually consistent set that experimentally establishes the S-V curve in the region of 4,000 K and rules out the possibility of an S-V curve in Fig. 1A. With the S-V curve thus established, the only other way to get an S-L-V triple point at $\sim 10^7$ Pa would be for Bundy's data to be in error by $> 1,000$ K, which is highly unlikely.

If the S-L-V triple-point pressure of carbon occurs at $\sim 10^5$ Pa, $\sim 10^5$ Pa, then it is reasonable to question the significance of the results obtained at $\sim 10^7$ Pa. The pressure reported range from 1.01 to 1.22×10^7 Pa, but most values approximate $1.01 - 1.03 \times 10^7$ Pa. Temperatures range from $\sim 3,970$ to $4,390$ K with no tendency to cluster about a particular value. All these high-pressure results have at least two major shortcomings: (1) in all cases, it was assumed that the carbon vapour pressure equilibrated with the pressurising gas. This does not necessarily happen, and some means must be provided to show that this condition has been achieved. Therefore, the pressures are suspect as they are not really experimentally determined but essentially assumed values. (2) No spectroscopy was carried out on the radiation that arrived at the pyrometer. It must be confirmed that line or band emission is not causing spurious pyrometer readings and it must be shown that the radiating gas surrounding the specimen is not optically dense. In the laser heating experiments of Whittaker *et al.*, much spectroscopy was carried out on the solid and gas phases. It was found that the carbon gas emits only Swan radiation. If impurities are present, additional lines or bands appear in the spectrum; hence, it is important to ensure that the pyrometer uses a wavelength range that is free of this radiation. Also, the optical density of the gas was found to increase with pressure, and, at $\sim 10^6$ Pa, the effect of the optical density is no longer negligible. At 10^7 Pa, the optical density would be quite high. Hence, a pyrometer may not be able to 'see' the surface of the condensed phase at this pressure. In this case, the reported temperatures would have little meaning. Indeed, the effect of high optical density could account for the wide range of reported temperatures.

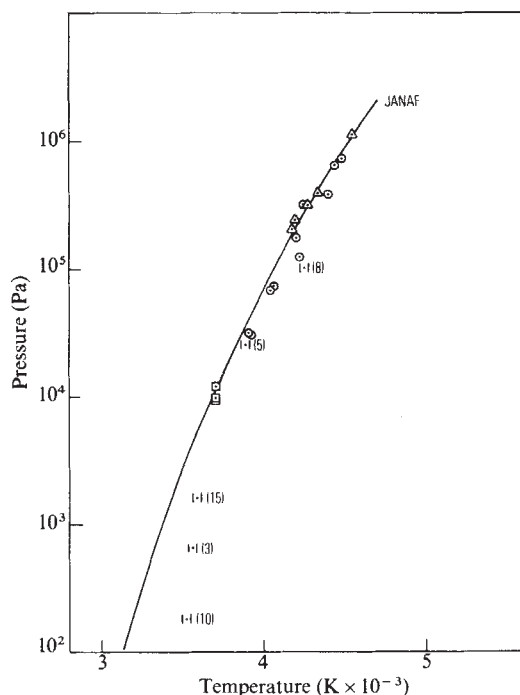


Fig. 2 Vapour-pressure data for carbon in the region of the S-L-V triple point. Δ , ref. 6; $\odot$, ref. 5; $\square$, ref. 7; $|-|$ (), refs 8-10, with error bars and number of determinations shown.

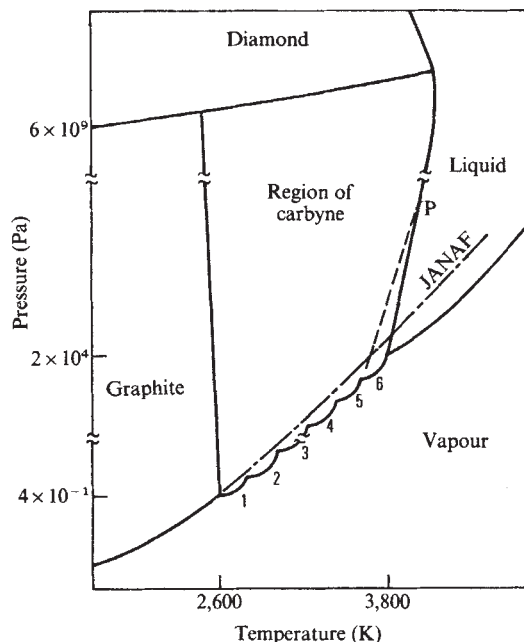


Fig. 3 Proposed carbon-phase diagram¹⁰.

One possible reason that the high-pressure data clusters in a general region is that a solid-solid-liquid triple point could exist near 10^7 Pa at point P in the proposed phase diagram (Fig. 3)¹⁰. In this case, the solid phases would have to be carbyne forms of carbon. As the equipment used in the high-pressure work was not designed to study condensed-phase equilibria, the observed pressure and temperature would be some kind of average determined by the competition between the condensed-phase equilibrium and the liquid-vapour equilibrium attempting to coexist. This situation would result in a large scatter in reported temperatures and pressures because this competition would depend on the idiosyncrasies of each particular apparatus.

The uncertainties discussed here make it difficult to interpret the high-pressure results. However, the recent vapour-pressure data together with Bundy's high-pressure results, contribute to the conclusion that the S-L-V triple point of carbon occurs at a pressure of the order of 10^5 Pa, and that the reported results in the region of 10^7 Pa must refer to some other phenomenon.

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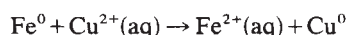
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A surface related component of iron metal in lunar samples?

SOLAR wind elements implanted into lunar soil grains come to rest within a few hundred angstroms, consequently high concentrations should build up in near surface locations. It has become common practice to 'identify' surface correlated species by investigating sieved soil fractions; the increased concentrations in fine grain sizes being attributed to the greater surface area. The approach used¹⁻⁴, infers a surface location for finely divided iron metal (Fe(0)) and associated hydrolysable carbon (C_{hyd}) in lunar samples. These studies, together with other evidence⁵, imply solar wind induced reduction processes were involved in producing metal from ferrous iron (Fe(II)) in silicates. The experiments described here performed with a reagent (CuCl₂/2KCl), which should only attack exposed metal, have failed to recognise a genuine exposed surface component. We believe that some effect associated with solar wind sputtering is responsible for iron formation and we must therefore question the validity of the model on which the initial conclusions in favour of surface located metal were based.

To obtain a quantitative measure of surface located iron metal, a mild specific leaching agent was used. Although X-ray photoelectron spectroscopy (XPS)^{6,7} may distinguish between Fe(II) and Fe(0) at depths <20 Å, this technique is not quantitative. Fe(0) is readily dissolved in a CuCl₂/2KCl solution due to the replacement reaction^{8,9}:



Two gram aliquots of tachylite basaltic glass (Snake River, Idaho) together with 0–5 mg quantities of iron metal (high purity, British Chemical Standards) were treated with 25 ml of an aqueous solution of the reagent (13.33 g CuCl₂ · 2H₂O, 11.66 g KCl in 250 ml doubly distilled water); filtered solutions were diluted so that iron was in the 0–1 p.p.m. range and analysed by atomic absorption to provide a calibration graph. A comparison with results obtained by acid treatment of iron turnings only, suggests that the copper reagent dissolves Fe(0) completely within the error limits of the measurement. The copper solution does not leach Fe(II) from the basaltic glass. Milligram aliquots of three suites of size differentiated glassy agglutinates separated from lunar soils 15601 and 12023 by sieving and density-magnetic methods^{2,10} were treated with 2 ml quantities of reagent. After hand shaking intermittently during a two hour period or standing over night, the liquid was directly aspirated into the flame of the atomic absorption spectrometer. Figure 1 compares the measurements of the amount of Fe(0) taken into solution with the total abundance of metal (diameter <130 Å) determined by magnetic susceptibility^{2,10}. With the exception of one sample of 15601 (150–250 μm fraction), the iron abundance in solution is small, close to the background or detection limits of the analytical method. There is no relationship with particle size; the single positive result could be attributed to dissolution of an iron meteorite fragment(s). The reagent is capable of dissolving Fe(0) associated with the samples since a variable quantity, up to 20% of the total, is found in solution after fresh CuCl₂/2KCl solution is added and agitated by ultrasonic vibration for 15 min (only sample 12023 *M* > 3.5 is shown in Fig. 1). Even after ultrasonic treatment, the amount of Fe(0) taken into solution does not increase with decreasing particle size; if anything our limited data suggest the opposite.

We interpret the above results as follows: the vast majority of Fe(0), possibly >99% of the total, is located in sites inaccessible to the reagent and not on the very surface of the samples studied. It could be argued that metal was present in cavities, vesicles and pore spaces¹¹ which could not be penetrated by the liquid. The analysis of solutions obtained using ultrasonics, however, are not in keeping with such a hypothesis, as very fine fractions do

not release the largest quantities of metal even after prolonged agitation. A microscopic examination of 12023 samples following ultrasonication shows that the agglutinates have been broken up; probably the iron was exposed to the reagent as a consequence. We would expect large agglutinates to be more fragile than small ones. For some reason, not yet clear, 15601 agglutinates were not disrupted by ultrasonic vibration.

Several mechanisms have been proposed for the production of lunar Fe(0) metal (for a review see refs 11 and 12). The hypotheses currently receiving the most attention involve reduction by some aspect of sputtering and by definition produce metal only at the very surface of grains. At face value, the results we have presented apparently preclude sputtering. They seem to favour a chemically based mechanism, whereby reduction is induced by meteorite impact heating, because water is formed from silicate oxygen and solar wind hydrogen atoms¹³. This latter mechanism might generate surface correlated metal which was inaccessible to the copper reagent. There is very strong evidence that a physical (sputtering) rather than a chemical reduction is involved: (1) both preferential sputtering of oxygen atoms⁶ and preferential deposition of heavy elements, particularly iron¹¹, are possible in practice; preferential sputtering is theoretically predictable¹²; (2) XPS and Auger spectroscopy demonstrate major elements at particle surfaces are fractionated relative to their abundance measured for the interior^{7,14}; and (3) silicon and oxygen at grain surfaces are depleted in light isotopes relative to these elements at greater depth¹⁵. Because of the apparent paradox, it is necessary to reconsider whether increased abundance of Fe(0) in fine fractions may only be interpreted as the presence of a surface related component.

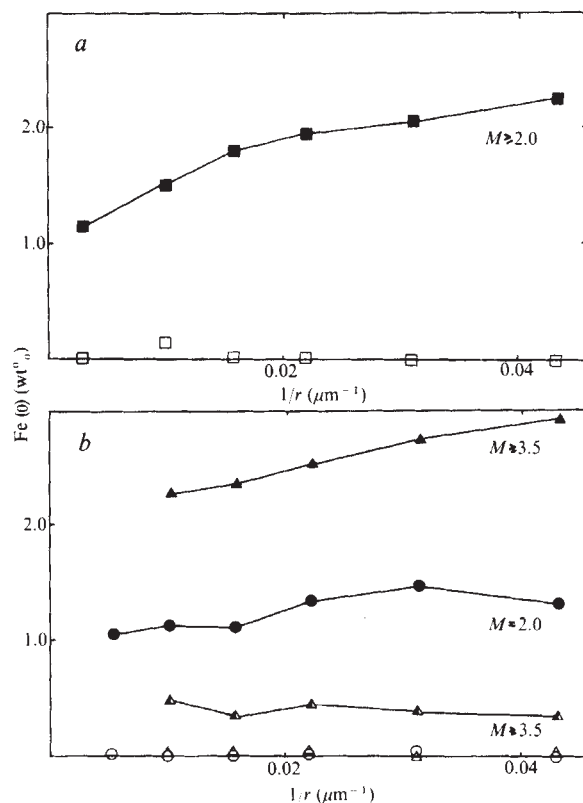


Fig. 1 Metal content of sieved fractions of glassy agglutinates from lunar soils: a, 15601; b, 12023. Closed symbols (■, ▲, ●) are for magnetic susceptibility measurements, open symbols (□, △, ○) are for CuCl₂/KCl leach determinations and ▲ refers to material agitated by ultrasonic vibration whilst in the liquid reagent. The values *M* = 2.0 and *M* ≥ 3.5 are the setting on the magnetic separator used to obtain the agglutinates from bulk soil; here they only identify the different fractions.

Figure 1 shows the data plotted as c against $1/r$ (where c = concentration and r = average radius of a size fraction). According to one model¹⁶ a surface component is recognised from the slope of the line; the volume related component can be determined for particles of infinite radius from the intercept on the y axis. In this model the volume related component is assumed to be constant for all grain sizes. Such an assumption is invalid because lunar soils contain many, in fact sometimes a majority of, grains which are complex aggregates, either glassy agglutinates or microbreccias. The volume related component is due to the incorporation of a surface component into the interior of complex particles. There is no reason to suppose a volume component produced in this way would be constant for all grain sizes. On the contrary, it might be expected that fine agglutinates and breccias, which must be made up of constituent fragments with a smaller median grain size than their coarse counterparts, ought to have a larger volume related component due to the increased surface incorporated. A volume component which increases in fine fractions would be indistinguishable from a surface component on a plot of c against $1/r$. An alternative model¹⁷ which considers $c \propto d^{-n}$ (where d is diameter) and recognises surface and volume related components from a plot of $\log c$ against $\log d$ is also incapable of distinguishing a surface from an increasing volume component.

We now contend that surface related components of Fe(0) and C_{hyd} are minimal; c against $1/r$ or $\log c$ against $\log d$ plots recognise increasing volume related components, which are a result either of a greater abundance of aggregated particles or a greater complexity within such grains in fine fractions. Some support for the latter interpretation has come from detailed analysis of progressively more magnetic agglutinates from lunar soil 12023 (ref. 10). We have pointed out^{2,12,18} that genuine surface components of Fe(0) < 130 Å in size would be unlikely to survive in the terrestrial atmosphere and that only metal incorporated into agglutinates would be protected from oxidation. Agglutinate metal behaving as an apparent surface related component because of increases in volume concentration does not conflict with such arguments.

If model predicted surface components of Fe(0) and C_{hyd} are in fact volume components, what proportion of the apparent surface correlated solar wind hydrogen, rare gases, carbon and nitrogen can be accounted for by this effect? The answer to this question presumably will come from comparison of data from size fractions with measurements made by surface specific techniques such as ion microprobe^{19,20} and nuclear reactions^{21,22}. Calculations²³ and direct measurements²² suggest maximum surface carbon concentrations are up to a factor of five less than estimates made on the basis of $1/r$ plots²⁴ and thus support arguments in favour of an increasing volume component.

We are not denying the existence of surface components, only questioning their magnitude; surface components must exist to be incorporated into complex grains to generate volume related components. We need better models to describe the accumulation of solar wind elements and solar wind derived species in lunar soil. Experiments using surface specific techniques to detect genuine surface components are highly desirable.

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Organic ¹⁴C activity in an abyssal marine sediment

ONE measure of transport rates and residence times of organic carbon within the various organic carbon pools in the ocean is to follow perturbations from the 1952–58 and 1961–62 atmospheric thermonuclear bomb tests on the natural ¹⁴C activities of living, detrital and dissolved organic matter. We report here our measurement of the ¹⁴C activity of the total sedimentary organic carbon (SOC) in an abyssal red clay, as no such data were available for this organic carbon pool. Ultimately, these sedimentary organic ¹⁴C activities may be compared with the ¹⁴C activity of the source organic material in the water column and estimates made of the magnitude of organic carbon consumption at the seawater–sediment interface. What was found, unexpectedly, was evidence for the penetration of recent organic carbon from the 4 cm mixed layer at the sediment–seawater interface down to at least 12 cm in the sedimentary column. This suggests a different and more rapid mechanism for the sedimentation of some fraction of the SOC other than direct association with clay minerals.

Photosynthetic fixation of bomb-derived ¹⁴CO₂ in the euphotic zone and its subsequent incorporation into bathypelagic marine fish and crustacea and into deep-sea particulate and dissolved organic matter has been previously studied in the north Central and North-east Pacific Ocean^{1–4}. This work (Table 1) showed that the dissolved (plus colloidal) organic carbon (DOC) had 'apparent ages' of 740 yr BP at the surface to 2,570–3,470 yr BP at 2,000 m, and that the ¹⁴C activity of the particulate organic carbon (POC) at 2,000 m was 10% less than its activity at the surface. These observations are indicative of slow recycling of DOC between the surface and deep waters and rapid sinking of POC (or some fraction of it) in the water column. Thus, high ¹⁴C activities of SOC in the upper mixed layer and at the seawater–sediment interface would reflect this rapid sinking of POC to the sea floor coupled with its relatively slow biochemical oxidation to CO₂ by benthic populations.

The 0–30 cm sediment sections (Table 2) were sub-sampled from two box cores⁵ (0.25 m² in area) taken on 10 July 1970 (core 33) at 6,040 m in the central North Pacific Gyre (30°0.9' N, 156°12.5' W) and on 8 June 1977 (core 638) at 5,780 m in the same area (28°34.4' N, 155°30.3' W). This region is part of an extensive abyssal plain situated beneath low-productivity surface waters. The sediment–seawater slurry (equivalent to a

Table 1 Natural radiocarbon activities of various carbon pools in the central and eastern North Pacific Ocean

Pool	Depth (m)	Date	Location	$\Delta^{14}\text{C}$ (‰)	Age (yr BP)*
POC	10	1971 ³	30°00' N 140°00' W	+237 ± 24	—
POC	2,000	1971	"	+182 ± 27	—
DOC	10	1971	"	-88 ± 20	740 ± 170
DOC	500	1971	"	-176 ± 14	1,560 ± 140
DOC	2,000	1971	"	-274 ± 13	2,570 ± 140
DOC	1,880	1968 ¹	30°16' N 119°49' W	-351 ± 27	3,470 ± 330
DOC	1,920	1969 ¹	30°16' N 119°50' W	-341 ± 23	3,350 ± 280
DIC	5,292	1973†	31°22' N 150°01' W	-219.5 ± 3.5	1,991 ± 46
SOC (this report)					
Core 33	6,040	1970	30°01' W	-664 ± 22	8,760 ± 510
(0–3 cm)			156°13' W	(mean)	(mean)
Core 638	5,782	1977	28°34' N	-736 ± 20	10,700 ± 630
(0–3 cm)			155°30' W		

POC, particulate organic carbon; DOC, dissolved organic carbon; DIC, dissolved inorganic carbon; SOC, sedimentary organic carbon.

*Ages calculated relative to the NBS oxalic acid standard activity using the Libby half-life (5,568 yr). Since pre-nuclear bomb surface seawater carbonates were 4‰ lower in activity ($\Delta^{14}\text{C} = -40\text{‰}$), all the ages given in this report are about 340 yr younger using this correction.

†Pacific Geosecs, Leg 1, Station 204 (H. Östlund, personal communication).

2-mm vertical section) was sucked-up from the undisturbed centre (25 cm × 26 cm area) of core 33 and sieved through a 65- μm mesh stainless-steel screen. The solid upper 3 cm of this core was then scraped into five arbitrary sections. The upper 3 cm of core 638 was sliced into 1-cm sections only as the surface of this core was slightly disturbed. The remainder of each core was sampled in 2-cm increments down to 30 cm leaving about 5 cm of sediment on the bottom. The samples were then frozen at -20°C for subsequent analysis. All feasible precautions were taken to prevent contamination of the samples with ^{14}C or dead carbon. The analytical results and a summary of experimental procedures for each analysis are given in Table 2. The $\delta^{13}\text{C}$ values are included to show that they fall within the range of marine organic- $\delta^{13}\text{C}$ analyses (-18 to -22‰ for subtropical waters).

Sedimentation rates below the mixed layer for the clay mineral fraction of cores 33 and 638, determined by $^{230}\text{Th}/^{232}\text{Th}$ activities, were 1.0 and $1.2 \text{ mm } 10^{-3} \text{ yr}^{-1}$, respectively (Fig. 1a). It is clear from this plot that the depth of the mixed layer is $8.0 \pm 1 \text{ cm}$ (core 33) and $4.0 \text{ cm} \pm 0.5 \text{ cm}$ (core 638), assuming uniform mixing within the layers. The 4 cm mixed layer thickness for core 638 is corroborated by the total SOC analyses (Fig. 1b). If SOC is laid down at the same rate as the clay minerals, then no measurable ^{14}C activity would be expected much below the mixed layer, however, significant ^{14}C activity was found down to at least 11–13 cm in core 638 (Table 2, Fig. 2).

The ^{14}C activity in the surface slurry (0–0.2 cm) from core 33 was not significantly higher than the ^{14}C activity found at 0.2–0.4 cm, and just significantly greater than the ^{14}C activity in the 2.7–3.0-cm section (Table 2). This implies rapid mixing and/or rapid utilisation in the mixed layer of POC reaching the seawater–sediment interface. The presence of bomb- ^{14}C in the mixed layers in cores 33 and 638 can be seen by calculating a hypothetical mean age of the SOC in the mixed layer⁹ assuming the $\Delta^{14}\text{C}$ value of the source organic carbon before the bomb tests was 0‰ (see Table 1). This calculation is based on the thickness of the mixed layers, M (8.0 and 4.0 cm), the $^{230}\text{Th}/^{232}\text{Th}$ sedimentation rates, S (1.0 and $1.2 \text{ mm } 10^{-3} \text{ yr}^{-1}$) and the decay constant for ^{14}C , λ ($1.2449 \times 10^{-4} \text{ yr}^{-1}$) where the mean age $= 1/\lambda \ln [1 + (\lambda/S)M]$. For core 33 this calculated mean age is 19,200 yr and for core 638 it is 13,200 yr (vertical dashed line, Fig. 2). When these calculated ages are compared with the experimentally determined ages (Table 2, Fig. 2), it is apparent that more bomb ^{14}C has entered the mixed layer in core 33 than in core 638. This increased input of bomb ^{14}C in the

mixed layer of core 33 is also reflected by the 42% more $^{239+240}\text{Pu}$ that was found in the 0–3-cm section of core 33 than in the 0–3-cm section of core 638 (Table 2). The presence of $^{239+240}\text{Pu}$ activity in the mixed layer of both cores is evidence of the rapid sedimentation of high ^{14}C activity POC as the flux of $^{239+240}\text{Pu}$ is, in part, through association with sinking organic detritus¹⁰.

The main feature of the organic ^{14}C activity in the sediments is the presence of measurable ^{14}C activity below the mixed layer in core 638 (Fig. 2). An 'apparent sedimentation rate' of recent SOC, calculated from the ^{14}C activities at 6, 8 and 12 cm is $1.15 \text{ cm } 10^{-3} \text{ yr}^{-1}$, and $0.95 \text{ cm } 10^{-3} \text{ yr}^{-1}$ if calculated from the activities at 1.5, 6, 8, and 12 cm (Fig. 2). These rates are about 10 times those derived from $^{230}\text{Th}/^{232}\text{Th}$ ratios, but due to uncertainties in the ^{14}C activity determinations, the recent SOC rates could vary from 0.8 to $6 \text{ cm } 10^{-3} \text{ yr}^{-1}$. Thus, the introduction of recent organic carbon below the mixed layer is

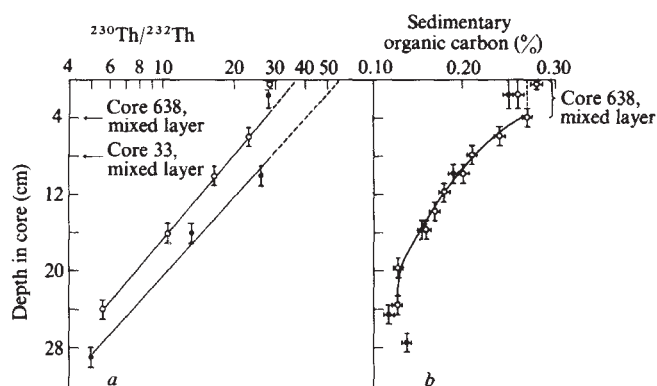


Fig. 1 a, $^{230}\text{Th}/^{232}\text{Th}$ ratios plotted as a function of core depth: ●, core 33; ○, core 638. Error bars denote one standard deviation. The lines are least squares fits and the depth of the mixed layers are indicated by arrows. The clay mineral sedimentation rates below the mixed layer are $1.0 \text{ mm } 10^{-3} \text{ yr}^{-1}$ for core 33 and $1.2 \text{ mm } 10^{-3} \text{ yr}^{-1}$ for core 638 where the half life of ^{230}Th is $7.52 \times 10^4 \text{ yr}$. b, Total sedimentary organic carbon (SOC) expressed as per cent of dry weight and plotted as a function of core depth: ●, core 33; ○, core 638. Error bars denote one standard deviation. The dashed line from 0 to 4 cm is the mean of values at 0–1, 0–3 and 3–5 cm for core 638.

independent of the sedimentation rate of the clay minerals or SOC associated with them.

The mechanism or mechanisms responsible for this apparently anomalous injection of significant quantities (calculated to be 7–13% of the total SOC depending on whether the source carbon is pre-bomb, $\Delta^{14}\text{C} = 0\%$, or post-bomb, $\Delta^{14}\text{C} = +180\%$) of recent SOC into the sedimentary column below the mixed layer is not clear. One explanation is organic carbon transport by burrowing organisms such that the numbers and lengths of their excursions, and hence the organic carbon flux, decreases with depth. The long straight burrows, 10–30 cm in length, which have been observed in abyssal sediments¹¹ are due primarily to worms burrowing into the sediment. The worms pump in surface seawater, filter out organic matter and eventually retreat leaving organic tubules behind. This mechanism for organic carbon transport is questionable for two reasons: (1) the smooth distribution of thorium isotopes (Fig. 1a) do not reflect any large scale bioturbation, and (2) the number of burrows normally observed in deep sea sediments do not seem sufficiently dense^{12,13} to maintain the necessary flux of recent organic carbon.

A second explanation for the injection of organic carbon into the sediments is diffusion of recent DOC into the sedimentary column from the mixed layer. This mechanism would require a negative gradient of DOC in the interstitial water and a high concentration of DOC to be continuously added to the mixed layer by relatively rapid biochemical processes (solubilisation of detrital POC, autolysis of organisms, and excretion of DOC by organisms). However, the opposite mechanism is indicated by an analysis of DOC in the interstitial water of a pelagic clay from the central equatorial Pacific¹⁴ (10°40' N, 147°40' W) where the DOC concentration increased from 2.5 mg C l⁻¹ at the surface to 4.8 mg C l⁻¹ at 30 cm in this core. Because the amount of DOC in the interstitial waters of red clays is only 0.1–0.4% of

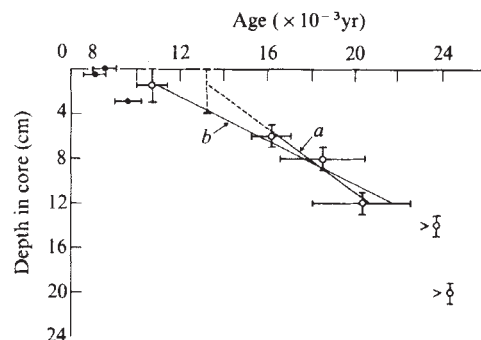


Fig. 2 The ^{14}C activity of the sedimentary organic carbon plotted as ages (yr BP) against core depth: ●, core 33; ○, core 638. Error bars denote one standard deviation (Table 2). The symbol (>) means that the ^{14}C -organic carbon activities at 13–15 and 19–21 cm were within 2σ of background and hence error bars were not applicable. The lines *a* and *b* are least squares fits for points at 1.5, 6, 8 and 12 cm; and at 6, 8 and 12 cm, respectively. The vertical dotted line from 0–4 cm represents the calculated age of the sedimentary organic carbon in the mixed layer for core 638 (see text).

the total SOC, unrealistically high diffusional fluxes of DOC would be required even with strong, negative DOC gradients. Thus, neither of the above mechanisms satisfactorily explains the presence of recent organic carbon 6–12 cm below the mixed layer in this pelagic red clay.

There is some corroborative information on possible bioturbation below the mixed layer from $^{239+240}\text{Pu}$ analyses of two box cores collected on the 1977 cruise. These two cores were taken 1.6 and 6.4 km from core 638. The $^{239+240}\text{Pu}$ analyses (V.

Table 2 Measurements made on the sediments and sedimentary organic matter from box cores 33 and 638

Sections (cm)	SOC (%)	H ₂ O (%)	$\delta^{13}\text{C}$ (‰)	$\Delta^{14}\text{C}$ (‰)	Age (yr BP)	$^{230}\text{Th}/^{232}\text{Th}$	$^{239+240}\text{Pu}$ (d.p.m. kg ⁻¹)
Core 33 (1970)							
Surface slurry (0–0.2)	0.20		–19.7	–655 ± 22	8,500 ± 500		
0.2–0.4	0.30		–19.7	–635 ± 23	8,100 ± 500		
2.7–3.0	0.27		–18.7	–703 ± 22	9,700 ± 600		
0–3		57				27.3 ± 0.1	1.7 ± 0.08
9–11	0.19	51				26.0 ± 1.3	
15–17	0.16	50				13.2 ± 0.4	
24–26	0.12	49					
28–30	0.14	49				4.9 ± 0.2	
Core 638 (1977)							
0–1	0.28	53				27.8 ± 2.2	
0–3	0.26	53	–20.1	–736 ± 20	10,700 ± 600		1.2 ± 0.16
3–5	0.27	52				23.0 ± 1.8	
5–7	0.24	51	–18.3	–866 ± 15	16,200 ± 900		
7–9	0.21	51	–20.3	–900 ± 21	18,500 ± 1,900		
9–11	0.20	50				17.6 ± 0.7	
11–13	0.18	49	–21.0	–920 ± 20	20,300 ± 2,200		
13–15	0.17	50	–21.1	–948 ± 0	> 23,700		
15–17	0.16	49				10.2 ± 0.2	
19–21	0.13	49	–21.3	–952 ± 0	> 24,300		
23–25	0.13	50				5.6 ± 0.3	

The thawed sediment samples used for ^{14}C activity determinations were acidified to pH 3–4 with distilled 6 M HCl, and all samples were dried to constant weight at 80 °C. Thereafter, the dried sediment samples were burned at 800–900 °C for 2–3 h in a stream of prepurified oxygen. Conventional vacuum line techniques were used to obtain CO₂ of high purity necessary for gas-proportional counting. Each CO₂ sample was aged for 4 weeks before counting to ensure decay of any ^{222}Rn (3.8-d half life). The ^{14}C activity was measured using a 100 ml quartz gas counter filled to a pressure corresponding to 90 cm CO₂ at 25 °C. In these conditions, 95% of the activity of the NBS oxalic acid standard was 0.727 ± 0.015 c.p.m. above a background of 0.815 ± 0.012 c.p.m. A minimum of 10,000 counts was accumulated for each sample, normally over two separate counting periods. Two blanks were run; one using no sediment and the other using an oxidised portion of the original sample. No CO₂ was evolved by either procedure. The CO₂ recoveries were 70–90% based on the total SOC contents. The $\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$ values and ages quoted in Table 2 have been calculated in the normal manner⁶. The $^{230}\text{Th}/^{232}\text{Th}$ (ref. 7) $^{239+240}\text{Pu}$ (ref. 8) methodologies were applied to the same non-acidified, dried sediment samples as used for the ^{14}C determinations, and the SOC contents were done using a Hewlett-Packard CHN Analyser. No differences were found in SOC between acidified and non-acidified sediment samples within the error of the method ($\pm 0.015\%$ C), indicating that the carbonate content of the sediment was <0.03%. This was confirmed by infrared analysis of the CO₃²⁻–CO₂ in non-acidified sub-samples.

Bowen, personal communication) showed penetration of $^{239+240}\text{Pu}$ down to 12 and 14 cm in these cores. Previous measurements¹⁵ by Bowen for two box cores taken in 1974 in the same general area showed penetration of $^{239+240}\text{Pu}$ from the surface down to 5 and 8 cm in the sedimentary column.

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Did emerging continents trigger metazoan evolution?

A DIVERSE fauna of trilobites, archaeocyathids, and other shelly invertebrates can be found in rocks of early Cambrian age, but in strata antedating the Cambrian such skeletonised remains are notably absent. This seemingly abrupt appearance of multicellular animals seemed illusory to Darwin¹, whose vision of evolution assumed the gradual transmutation of species over innumerable generations. His view of life necessitated a long Precambrian record of evolving Metazoa and he, therefore, attributed the apparent absence of Proterozoic fossils to the imperfection of the fossil record and the vagaries of sedimentary geology. Discoveries of the past two decades have shown the first metazoans to be less ancient than Darwin believed. It now seems that the Metazoa evolved only in the latest Precambrian (perhaps 750–650 Myr ago), and that 100–150 Myr of evolution was sufficient to delineate the various phyla in evidence at the base of the Cambrian. The question of whether emerging continents controlled the late appearance of multicellular animals on Earth is discussed here.

Hargraves' theory² of crustal evolution has inspired new hypotheses on metazoan origins. Hargraves believes that the primitive Earth had a thin, but continuous, sialic crust blanketed by a world ocean some 2.8 km in depth. As the Earth matured, mantle activity progressively built the sialic crust into continental masses. The thickness of these continents was controlled isostatically, the critical crustal depth being the 750 °C isotherm (the temperature at which granitic crust begins to melt). As long as the Earth's interior was hot and, consequently, the 750 °C isotherm was shallow, the continents could not become thick enough to rise above sea level. Only as the Earth cooled could continental blocks emerge from and dissect the world ocean. Hargraves sets wide limits on the timing of continental emergence. His geophysical model for terrestrial cooling predicts that continents should have appeared 1,400–900 Myr BP, while his estimate of sedimentary rock volumes through time suggests that the actual time of emergence was 900–600 Myr BP. Hargraves does, however, acknowledge that certain patterns of sedimentation are most consistent with the existence of emergent continents before 1,700 Myr BP.

Chamberlain and Marland³ were the first to consider the biological implications of Hargraves' theory. If Hargraves is correct, they reasoned, then before the buildup of continents to within 200 m of sea level, the global sea would have been stratified. Nutrient recycling by upwelling would have been minimal, and consequently, organic productivity would have been low. Chamberlain and Marland link both increased algal productivity and metazoan evolution to the appearance of persistently emergent continents.

LaBarbera⁴ has taken these arguments further and developed an explicitly hargravesian theory of metazoan origins. He argues that it was the emergence of continents in the late Precambrian that triggered the evolution of multicellular organisms. Before the end of the Proterozoic era, environments in which early invertebrate benthos could proliferate simply did not exist, except during a few transitory periods.

If we wish to accept the theories of metazoan evolution implied by Chamberlain and Marland and defined by LaBarbera, we must first examine the physical model on which they are based. Instead of judging the merits of the Hargraves' model in its entirety⁵, I shall concentrate on the aspect that bears directly on the derivative biological arguments—the timing of the appearance of continental land masses. If sizeable continents with their associated implications for erosional runoff, shallow marine platform environments, and upwelling currents existed significantly before the end of the Precambrian, then it is difficult to attribute the evolution of Metazoa to their appearance. The appropriate data for the examination of this question are the preserved accumulations of Precambrian sedimentary rocks. From these strata, one can derive important information about the type, depth, and extent of depositional environments, as well as data relevant to the delineation of sediment source areas.

The Proterozoic sedimentary record of North America is relatively well known and illuminates the problem. The oldest Proterozoic sediments on this continent are those of the Huronian Supergroup exposed north of Lake Huron. This 2,300 Myr old sequence, which has an aggregate thickness of ~15,000 m near the southern limits of exposure, contains a variety of features suggestive of deposition on the margin of a large, emergent land mass: deposition on a pre-Huronian erosion surface, sedimentary structures indicative of fluvial deposition, other structures referable to tidal flat environments, clasts derived from a granitic source area to the north, and a set of characteristics (striated pavement and boulders, tillite, and dropstones) that record an episode of early Proterozoic glaciation^{6–9}. Sediments of similar age and character are also known from other areas of North America: the Kaminak Basin, North-West Territories; the Chibougamau Basin, Quebec; and the Libby Creek Group of Wyoming¹⁰.

Sedimentary rocks deposited between 2,200 and 1,800 Myr BP are common in North America, occurring in linear geosynclinal belts and as the remnants of broad platform sequences.

Goodwin¹¹ has shown that the geographical distribution of these deposits is consistent with the occurrence of two large, stable early Proterozoic cratons separated by a metastable cratonic area. Goodwin's continental areas are bordered on all sides (within the limits of existing outcrops) by miogeosynclinal sedimentary rocks. Within these facies, there is abundant physical evidence of shallow water sedimentation. For example, in the miogeosynclinal belt of the Coronation Geosyncline, North-West Territories one finds arkose and orthoquartzite (derived from a source located in the direction of Goodwin's hypothesised craton), oolites, mudcracks, and gypsum casts¹². Similar features characterise the proximal facies of the CircumUngava Geosyncline in eastern Canada¹³⁻¹⁵. Contrary to the assertion by LaBarbera, stromatolites abound in early Proterozoic carbonate sequences. The abundance and variety of stromatolitic structures in Coronation¹⁶ and Circum Ungava^{17,18} shelf sediments, as well as in other coeval deposits (see Table 1), provide strong testimony to the wide distribution of shallow marine environments 2,000 Myr ago. These environments were not the transitory products of dynamic crustal features such as island arcs.

Table 1 Occurrences of early Proterozoic stromatolites

Geological unit	Location	Refs
Gunflint/Biwabik Fms	Lake Superior, USA and Canada	46, 47
Kona Fm.	Michigan, USA	48, 49
Great Slave Supergroup	North-West Territories, Canada	50, 51
Hurwitz Group	North-West Territories, Canada	51
Sutton Lake Group	Ontario, Canada	51
Manitounuk Group	Quebec and Labrador, Canada	51
Belcher Group	Belcher Islands, Hudson Bay, Canada	13-15, 51
Mistassini Group	Quebec, Canada	52
Vallen Group	Southern Greenland	53
Mt Bruce Supergroup	Western Australia	54
Fortescue Group		
Hamesley Group		
Wyloo Group		
Frere Fm.	Western Australia	55
Batchelor Group	Northern Australia	30
South Alligator Group	Northern Australia	30
Ventersdorp Group	South Africa	56
Transvaal Supergroup	South Africa	26
Lomagundi Dolomite	Rhodesia	57
Karelian Series	Soviet Karelia, Finland	58
Burzyan Group	Southern Urals USSR	59
Upper Dharwar and Aravalli Groups	India	60

They are thick sequences resting on stable Archean granites and gneisses. In short, it is difficult to account for the observed pattern of early Proterozoic sedimentation without postulating the existence of large emergent or periodically inundated continental masses.

The mid-Proterozoic history of North America strengthens the case for the early appearance of continents. Between 1,700 and 1,000 Myr BP, several grabens developed at various times and in various parts of the Canadian Shield¹⁹⁻²¹. These basins were filled with continental redbeds and basalts in a way similar to that characterising the Triassic-Jurassic rift basins of eastern North America. In the North-west Territories, one also finds evidence of a >1,500 Myr old blanket sandstone (the Thelon Formation and its equivalents)¹⁹ that seems to be similar in nature to the Phanerozoic 'Nubian' Sandstones of Africa. Palaeocurrent analysis of this formation suggests sediment derivation from an actively eroding land area in the vicinity of Hudson Bay¹¹. During this same period thick sequences of clastics and stromatolitic carbonates were accumulating on the eastern (Grenville)²² and western (Belt/Purcell)²³ shelves of the shield. Again, a large, emergent craton seems to be fundamental

to any explanation of mid-Proterozoic sedimentation patterns in North America.

The North American record is not unusual in its pattern of Proterozoic sedimentation. Indeed, data from other continents corroborate the picture of Precambrian continents inferred from North American rocks. Several episodes of epicontinental and continental sedimentation covered the Kaapvaal Craton of southern Africa during the early Proterozoic era²⁴. In earliest Proterozoic times, auriferous fluvial and alluvial clastics of the Witwatersrand Supergroup accumulated in a continental basin whose area was at least 70,000 km² (ref. 25). Stromatolitic carbonates of the overlying Transvaal Supergroup (2,200 Myr) suggest a shallow marine platform of still greater dimensions. Eriksson and Truswell²⁶ have traced the Malmani Dolomite throughout an area of 210,000 km². (Not only is this formation widespread geographically, it is also up to 1,500 m thick.) The Transvaal sequence itself is overlain by an extensive blanket of redbeds belonging to the Waterberg Group (1,800 Myr BP)²⁷.

Similar patterns can be found in Australia where broad platforms and geosynclines characterise early²⁸⁻³⁰, middle^{30,31}, and late Proterozoic^{32,33} sedimentary sequences. Palaeogeographical maps of the USSR for the early Riphean (1,650-1,350 Myr) illustrate emergent Russian Platform and Siberian shield areas flanked by broad expanses of shallow marine platforms³⁴. In South America, deltaic sediments are known from the earliest Proterozoic of Brazil³⁵, and 1,800-2,000 Myr old blanket sandstones are widely distributed across the Guyana Shield³⁶.

From the geological evidence, it seems that the observable record of Proterozoic sedimentation does not support the thesis that continents first became emergent late in the Precambrian era. Sizeable cratonic areas have been a persistent feature of the Earth for at least the past 2,300 Myr (I do not argue here that continents emerged at this time or that they ever emerged as envisioned by Hargraves, I simply state that they were there 2,000 Myr ago). The contention that continental areas existed in the early Proterozoic does not carry with it the implication that Hargraves' graph of sediment volume against time is necessarily incorrect. The apparent increase in sediment volume indicated for the Phanerozoic could be a real phenomenon related to Phanerozoic plate movements. Geological evidence—including cross-bedded quartz arenites, stromatolites, tidal flat sequences, and shales whose geochemistry suggests prolonged subaerial weathering—indicates that emergent land masses and shallow marine environments also existed in the Archean, although the extent and longevity of such features are less well defined than they are for the Proterozoic (see refs 37-39). I cannot agree, therefore, that Metazoa arose in response to the initial appearance of land on the Earth. LaBarbera's hypothesis that the first primitive herbivores and deposit feeders evolved from a pelagic planuloid ancestor is plausible, as is his contention that the subsequent evolution of predation greatly increased the complexity of the food web and led, through increasingly specialised adaptations, to the diversification of multicellular animals. But these steps need not have been triggered by emerging continents. They could equally well have taken place in pre-existing shallow water environments.

The concept that it was the removal of some physical barrier that made metazoan evolution possible is not new. A decade ago, the Berkner-Marshall hypothesis⁴⁰ gained widespread popularity because it provided a simple set of non-biological controls over biological innovation⁴⁰. But the physical constraints of oxygen and ozone, like those of habitat, seem to have been removed well before the first invertebrates populated the ocean floor⁴².

Why then did the Metazoa evolve when they did? I believe the answers are fundamentally biological in nature, rather than physical. As hypothesised by other workers, the rapid evolution of multicellular animals at the end of the Precambrian may have resulted from a combination of biological factors including the origin of eukaryotic sexuality, the ecological consequence of cropping, and the acquisition of that most basic of metazoan

characteristics, a biochemical mechanism for the functional differentiation of genetically identical cells⁴³⁻⁴⁵.

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Identification of ancient heat treatment in flint artefacts by ESR spectroscopy

It is of archaeological importance to be able to determine whether or not a particular sample of flint has been subjected to heating, either intentionally or accidentally, during its utilisation by man. We report here on the use of ESR spectroscopy for the study of flint, and show that heated material can be readily identified by the appearance of a characteristic ESR signal which is absent from the unheated flint. This signal is considered to be

stable over an indefinite period of time. We have also explored other implications of this technique in the examination of flint, including its possible use for dating purposes.

Worked flint is the most abundant source of evidence for human activity before the advent of pottery-making cultures, and any information which can be deduced from it regarding its technology and date of fabrication is of considerable archaeological interest. Excavations frequently produce flint which has been accidentally subjected to the action of heat in a fire, but it is now recognised that from at least the Solutrean (~17000 BC) some flint was intentionally heated to improve its working properties^{1,2}. Using experimental fabrication technique^{3,4} and examining artefact material⁵ show that the technique of pressure retouching is facilitated in certain flint samples by controlled heat treatment of the material, and that this fact was known and exploited in antiquity. Samples thus treated can often be recognised by the greasy lustre of fractures produced after heating, but because the useful heat treatment temperature is in the region of 300 °C, well below that at which gross structural changes become apparent, it is important to have a reliable method for identifying flint which has been heated. Flint which has been heated above about 380 °C may be suitable for dating by the thermoluminescent dating method^{6,7}, and some dates have been obtained for archaeological samples although there are still considerable problems associated with the application of this technique to flint which are not yet resolved. Thermoluminescence (TL) has been used to determine prehistoric heat treatment of chert artefacts⁸, and has proved successful for material heated within the past 10,000 yr. Caution must, however, be exercised with samples from earlier sites where the TL acquired since heating, approaches the saturation value for the unheated geological material, and the method is necessarily destructive.

The technique of ESR spectroscopy has been used to assess radiation damage in geological materials,⁹ and has recently been applied to cave deposits and bone associated with early human activity¹⁰, but it has not previously been used for artefacts. It is generally less sensitive than TL but can, in principle, lead to firmer identification of the radiation-induced centres.

Our results on unheated geological samples showed the presence of a very narrow readily saturated line ($g = 2.0017$, width ($\Delta H_{MS} = 1.0$ G), which we assign to trapped electrons (e^-). On heating to 400 °C for 15 min this signal was lost, and exposure to a 100 krad dose of ⁶⁰Co γ -rays at 300 K readily restored it. We conclude that qualitative study of this e^- signal could be a useful and complementary technique to TL in studies of flint artefacts, and we are attempting to establish quantitative links.

Most significantly, on heating, a new signal (C) ($g = 2.0035$, $\Delta H_{MS} = 3.5$ G) developed. This is characteristic of amorphous carbon^{11,12}. Traces of organic matter are known to be present in flint¹³, and we postulate that these pyrolyse on heating to give carbon. The paramagnetic defects responsible for the ESR signal are, as far as we know, infinitely stable at ambient temperatures. Hence the presence of a C-signal should be diagnostic of heat treatment. To test this, we studied a group of upper Palaeolithic flints (~20000 BC), two of which were thought on the basis of appearance to have been heated in antiquity. The former gave clear C-radical signals together with e^- features, whereas the latter gave only the e^- signal.

These results establish that we have discovered a well defined method of assessing the occurrence of heat treatment in archaeological flint samples. We are now initiating a quantitative study of the C-signal as a function of the firing temperature, the time of firing and the nature of the flints. Hence we hope that ESR studies can establish conclusive details of heat treatment, and may also help to establish the probable date of such treatment.

We have also detected various other ESR signals in certain flint samples, some of which are characteristic of transition metal ion impurities such as Mn(II) and Fe(III). The form of the spectra provide information about the local symmetry around these ions. By studying their response to exposure to ⁶⁰Co

γ rays, we should be able to gauge the extent to which these impurity ions contribute to radiation effects in flint. This could be significant as their participation could greatly modify TL in such samples.

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Botanical evidence linking the New Zealand Maoris with New Caledonia and the New Hebrides

TRADITIONAL history of the Maoris tells of repeated voyages between Hawaiki, their mythical homeland, and Aotearoa (New Zealand), culminating in a considerable migration about AD 1350 dated by genealogies. One canoe is said to have brought seed of the karaka tree which was a useful subsidiary food source. I report here that examination of the distribution and relationships of this tree suggests New Caledonia and/or the New Hebrides as the possible Hawaiki from which this late migration originated.

The tree *Corynocarpus laevigatus* J.R. et G. Forst. is commonly seen in the North Island of New Zealand and in the warmer coastal parts of the South Island to the southerly limit of Banks Peninsula. It is well known by its Maori name *karaka*, or on Chatham Islands where it is also commonly known by the Maori name *kopi*. Over its whole range along the coastline frequent groves are explained as Maori plantings marking old villages or campsites because in pre-European times the tree was important for food. The large kernels are poisonous but became valuable and highly nutritious when steamed or baked and washed to free them from the compound karakin which causes severe convulsions and usually death if eaten⁵.

Among the interesting stories of the ancient traditional history of the New Zealand race¹¹, there is the record that karaka berries were brought to New Zealand in the canoe Aotea, one of a group or 'fleet' supposed to have brought a large number of Maori immigrants about the year AD 1350 (ref. 3). Other Polynesian settlements had been established in New Zealand several centuries before this wave of colonisation^{3,7}. Botanists, nevertheless, have described the tree as an endemic species and it is generally regarded as such today¹, although its

distribution is not as widespread as might be expected had it been growing in New Zealand for millions of years.

Seed grows readily and is widely planted in gardens throughout the country. In the wild wherever plants are protected from stock which eat foliage and fruit, seedlings arise in profusion around old trees, although they do not all survive if heavily shaded. Native pigeons, *Hemiphaga novaeseelandiae*, are known to feed on ripe berries and probably void viable seeds which are protected by a hard coat, although pigeons in laboratory trials were killed by a small dose of extract of karakin or its hydrolysate². Occasional seedlings which have been noted some kilometres away from coastal groves may have been established by pigeon distribution, although pigeons are feeble fliers and if they retained seed for long they risk being poisoned. Maoris also planted seed along ridges and bush routes which they traversed, and groves may be found near old settlements growing from discarded seed thrown on middens¹³.

Cockayne, discussing the vegetation of New Zealand⁴, describes *C. laevigatus* as a leading physiognomic plant in coastal vegetation but also makes the important remark. "As Maoris were accustomed to plant this tree near their villages doubt must frequently arise as to its occurrence in certain localities not being due to this cause. Few plants spread more rapidly by means of seed so its presence in apparently primeval forest does not disprove its being absent in the primitive community." More detailed observations on the distribution of the karaka tree are necessary, but the firm impression remains from wide observation over its range that the species is absent from many places where it can grow very well, and where one would expect it to be were it truly indigenous. The distribution on the Kermadec Islands¹⁵ seems to be sporadic as it is in New Zealand, as though the Maoris introduced it rather than took it from there, as has been suggested³.

Hemsley¹² described another species of *Corynocarpus* from the New Hebrides as *C. similis* because it closely resembled *C. laevigatus*. A re-examination of this species has led me to the opinion that *C. similis* is in fact the same species as *C. laevigatus*. Two flowers of the holotype of *C. similis* can be matched in detail with flowers of *C. laevigatus*, especially in the structure and tooting of the stamens which Hemsley used as a distinguishing character. Additional flowering specimens of *C. similis* collected in the New Hebrides and fresh flowering material collected on the island of Efate are all in agreement. Leaves of the New Hebridean plants have a slightly larger maximum size and are distinctly thinner, but venation, epidermis and the finely down-rolled margin correspond with New Zealand specimens. Inflorescences are also more open and lax. Such vegetative differences may warrant making the New Zealand and New Hebridean plants different varieties of the same species, but the exceedingly close relationship between the two is certain and obviously is of special interest in connection with the Maori tradition of the origin of the karaka in Hawaiki. Hemsley¹² also described a new species, *C. dissimilis*, from New Caledonia. Careful examination shows flowers and foliage of this to be so close to material of *C. laevigatus* that it is assumed to be conspecific.

It seems improbable that the natural distribution of this single species should cover the New Hebrides, New Caledonia and New Zealand, in the light of distribution of other species of the New Zealand flora and the highly endemic character of its trees. The heavy, bulky, poisonous seed sinks in water, so that recent natural distribution over such a distance seems to be ruled out. *C. similis* is known to be present on all the main New Hebrides islands and has a vernacular name on each, seeming, therefore, to be a truly endemic plant of the group: none of the vernacular names listed by Gowers⁹ bears any resemblance to the Maori names karaka or kopi. The genus is unknown from Fiji and Samoa, which are both well explored botanically. One other distinct species extends from Malaysia to Queensland¹⁴.

Some early link between the New Hebrides, New Caledonia and New Zealand is strongly suggested by this botanical evidence, for some trees are clearly very old. As they do not form

annual rings age cannot be counted in the usual way. Yen¹⁶ has written of the movement of the sweet potato, *Ipomoea batatas*, from South America to Oceania, and the survival of the Peruvian, Ecuadorian and Bolivian name kumara. Although direct evidence of the people who effected the transfer has been lost, it is generally considered that they were probably Polynesian voyagers making early contact with South Americans between AD 400 and 700. If ocean travellers had collected the karaka in a similar manner it should be present throughout Polynesia in places other than just the New Hebrides, New Caledonia and New Zealand. This distribution supports the suggestion of direct migration, from New Caledonia and/or New Hebrides to New Zealand, based on two-way voyaging which would have suggested that an otherwise perhaps unimportant food plant would be of more use in the new land.

New Zealand anthropologists today discount the story of the 'fleet'¹⁰, but the older suggestion that this traditional history has a factual basis is still accepted by many, including myself. Although it is clear that the links of the earlier Polynesian settlers who arrived in New Zealand from about AD 900 are all with east Polynesia^{3,7}, the evidence of the karaka suggests that later Polynesian settlers who arrived about AD 1350 came from New Caledonia and/or the New Hebrides, which are presumed to have contained Polynesian settlements, about 600 yr ago. They are now inhabited by Melanesians, although Polynesians and Polynesian languages are found in the New Hebrides. The large area of these islands and their fine timber resources could have supported a considerable population of seafaring people. Instructions in the traditional history speak repeatedly of the necessity of steering to the east when sailing from Hawaiki to Aotea (New Zealand). The expert seamanship and navigational ability of the Polynesians, appreciated by Captain James Cook and all the early European navigators in the Pacific, have been well examined recently^{6,8} and can be in little doubt, although questioned by some¹⁰. The fighting referred to in the traditional history, which seems to have been a main factor driving the people of the later migration out of their Hawaiki, may have been with Melanesian invaders ousting the Polynesians, who fled to New Zealand in considerable numbers taking with them such western cultural features as the rich use of curvilinear designs and the production of many kinds of weapons, which are so characteristic of the later 'classical' Maoris but are absent from the Moa-hunter period.

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Humeral morphology of the earliest apes

KNOWLEDGE of the skeletal anatomy of early hominoids has been based on various skeletal parts of fossil apes from the Miocene of East Africa and Europe. Postcranial remains from the earlier apes of the Oligocene of Egypt consisted of a single ulna, a basal phalanx and a hallucial metatarsal¹⁻⁴. During 1977 a palaeontological expedition from the Cairo Geological Museum and Duke University recovered four new partial humeri attributable to these Egyptian apes. These specimens now provide the first evidence of humeral anatomy of Oligocene apes and permit a better assessment of primitive forelimb morphology and locomotor abilities of earliest hominoids.

All four fossils were recovered from quarries in unconsolidated channel sands of the 'Upper Fossil Wood Zone', Jebel Quatrani Formation. This formation has been radiometrically dated as >26 Myr old⁵. All four bones are undistorted but broken so that they preserve varying portions of the distal third of the humerus. Each humerus is distinctly recognisable as primate, and because of differences in absolute size they seem to represent two taxa. Two of the specimens (DPC-1026, CGM-40123) are similar in size to humeri of the extant New World howling monkey *Alouatta*. These bones are almost certainly attributable to the largest of the Egyptian Oligocene apes, *Aegyptopithecus zeuxis*. Each articulates well with the previously described ulna of this species¹. Two other humeri (DPC-1033, DPC-1045), although similar in overall morphology to the larger fossils, are from a smaller animal the size of the bearded saki (*Chiropotes*) (Tables 1 and 2). There are two ape species smaller than *Aegyptopithecus* known from the quarries which yielded these humeri, the type species of *Aeolopithecus*, and a new undescribed species of *Propliopithecus*. It is not clear to which of these smaller species, if either, the latter two humeri are attributable.

In order to assess both their functional and their phylogenetic significance, the new fossil humeri have been compared with samples of over 30 species of living primates and a variety of nonprimate mammals using both metric and qualitative characters. Table 2 compares the Oligocene humeri with selected groups of extant species using indices which reflect the most distinctive features of the fossils.

Table 1 Characteristics of three humerus specimens of Oligocene apes

Specimen	DPC-1026	CGM-40123	DPC-1033
Bicipondylar width	34.1	—	23.4
Angular projection of medial epicondyle	8.6°	—	6.2°
Ventral articular width	24.5	22.9	17.0
Ventral trochlea width	11.5	11.3	8.2
Ventral capitulum width	12.5	11.5	8.9
Maximum capitulum depth	13.0	11.8	10.1
Olecranon fossa depth	5.5	5.6	4.3

All linear measurements are in millimetres.

In the form of the distal articular surfaces, the fossil humeri show none of the derived features found among modern cercopithecoid monkeys and lack many derived humeral features characteristic of extant hominoids. Rather, they show a more primitive mosaic of features similar to those found in Miocene fossil apes and some present-day New World monkeys. As in most primates, the capitulum is rounded rather than cylindrical, a shape which presumably permits extensive rotation of the radius about the ulna during pronation and supination. The surface of the capitulum extends well onto the inferior surface of the humerus, indicating that the early apes were capable of a considerable range of elbow extension⁶. Compared with the condition seen in cercopithecoids, the radial side of the humerus

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Table 2 Comparison of Oligocene humeri with extant species

Species	Capitulum depth	Trochlea width	Medial epicondyle	Angular projection of medial	Olecranon depth
	Articular (Index A)	Capitulum (B)	Articular (C)	(D)	Articular (E)
<i>Chiropotes satanas</i> (2)	0.614	0.614	0.271	44.0°	0.307
<i>Alouatta</i> sp. (7)	0.579	0.809	0.365	20.4°	0.196
<i>Macaca fascicularis</i> (7)	0.825	0.642	0.258	44.1°	0.392
<i>Cercopithecus aethiops</i> (7)	0.848	0.648	0.256	50.1°	0.422
<i>Cercopithecus mitis</i> (6)	0.789	0.661	0.288	42.6°	0.352
<i>Presbytis obscura</i> (3)	0.710	0.774	0.212	37.0°	0.373
<i>Hylobates</i> sp. (7)	0.692	0.868	0.295	6.0°	0.304
<i>Pan troglodytes</i> (5)	0.623	0.946	0.400	15.8°	0.346
<i>Dendropithecus macinnesi</i> (1)	0.575	0.920	0.342	0°	0.311
<i>Pliopithecus vindobonensis</i> (1)	0.524	0.842	0.314	0°	0.253
DPC-1026	0.530	0.920	0.351	23.0°	0.224
CGM-40123	0.515	0.983	—	—	0.245
DPC-1033	0.594	0.921	0.364	24.0°	0.252

Values of the indices are given as means.

Figures in parentheses represent the number of specimens measured.

is relatively shallow in the new fossils as in New World monkeys and other fossil hominoids (Table 2, index A).

Medially, a low ridge separates the capitulum from the trochlea. In these Oligocene apes, this ridge is much less marked than that found among living hominoids or in Miocene *Dryopithecus africanus*; it is more comparable in shape to that of the Miocene ape *Pliopithecus* or of larger cebids such as *Alouatta*. These humeri show the relatively broad trochlea found among all other extant and fossil hominoids. This feature separates them quite clearly from cercopithecoid monkeys (Table 2, index B). Since a broad trochlea is also found among many prosimians, as well as these Fayum specimens, its presence among modern apes is almost certainly a retention of a primitive condition.

The Oligocene humeri lack the distinctive spool-shaped trochlea with pronounced lateral and medial margins that characterises extant apes and the Miocene fossil ape *D. africanus*. This spool-like shape, Jenkins has argued, is biomechanically adapted for maximising elbow stability through an extensive range of flexion and extension as well as in pronation and supination⁷. Even so, these fossils do not possess a narrow trochlea with pronounced anteromedial and posterolateral borders such as is characteristic of cercopithecoid monkeys (and many other pronograde quadrupedal mammals)⁷. The broad relatively shallow trochlea of Fayum apes, with its slightly developed anteromedial lip, is more comparable to that found

among the more generalised arboreal quadrupeds such as the large cebids *Alouatta* or *Chiropotes*. In these New World monkeys the elbow is frequently flexed and the forearm semipronated during quadrupedal locomotion⁸.

In the Fayum ape humeri, there is a large medial epicondyle which projects slightly posteriorly (Table 2, C, D). Among extant apes and many prosimians this process is large and projects directly medially. Inward projection of the medial epicondyle increases the medial rotatory torques which the digital and carpal flexors, as well as pronators, exert about the radio-humeral and ulnar-humero joints⁷. Amongst most Old World monkeys the medial epicondyle projects more posteriorly than medially as an adaptation for enhancing the actions of the pronators and flexor muscles when the elbow is already pronated as in pronograde quadrupedalism. The condition found among the Fayum apes is similar to that occurring in the larger arboreal quadrupedal cebids such as *Alouatta* or *Lagothrix* and represents a biomechanically intermediate condition between that of Old World apes and of cercopithecoid monkeys. On the dorsal surface of the medial epicondyle is a pronounced dorsal epitrochlear fossa similar to that seen in *Alouatta* and *Apidium*³.

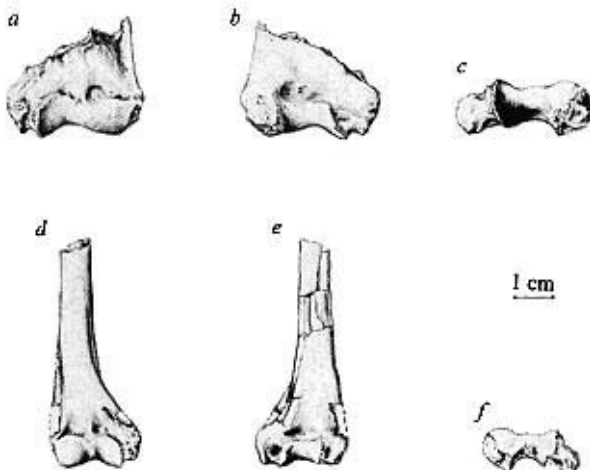
As in most extant prosimians and cebids, the olecranon fossa of Oligocene apes is quite shallow (Table 2, index B). In pronograde quadrupeds and knuckle-walking apes the olecranon fossa is deep. In function, a shallow olecranon fossa primarily appears to reflect the reduced posterolateral lip on the trochlea, and hence a reduced need for ulnar-humero stability during full extension of the elbow⁷.

Although only one specimen preserves more than the most distal part of the humeral shaft, all four humeri show an entepicondylar foramen. This foramen, which transmits the median nerve and brachial artery from the dorsomedial surface of the arm to the cubital fossa, is characteristic of most prosimians (except lorises) and many cebids. Among catarrhines it is known only in the parapathecoid genus *Apidium*³, the Miocene hominoid *Pliopithecus*⁹ and as a rare variation in other genera including *Homo*. Its occurrence in the Fayum hominoids confirms that its presence is the primitive condition for both higher primates and hominoids.

In all four humeri, there are indications of a prominent brachialis flange similar to that found among extant and fossil hominoids, as well as in medium-sized cebids and many climbing mammals. This flange is greatly reduced in Old World monkeys.

Few regions of the skeleton are more diagnostic for differentiating living monkeys and apes than the elbow region. The specimens described here provide new and unique palaeontological evidence about the morphology of this region in the earliest apes, that can be used to distinguish primitive from derived morphological conditions in living Old World higher

Fig. 1 Anterior (a), posterior (b), and distal (c) views of the left humerus of *Aegyptopithecus zeuxis* (DPC-1026) Anterior (d), posterior (e), and distal (f) views of the right humerus of a smaller hominoid (DPC-1033).



primates. The new fossils indicate that a large medial epicondyle, relatively broad trochlea, and a pronounced brachialis flange, all found in Fayum apes, in *Apidium*, in many platyrrhines and prosimians as well as extant hominoids are primitive characters. The development of the distinctive spool-shaped trochlea with a strong ridge delineating its lateral border is a derived condition unique to extant hominoids. Such features of present day Old World monkeys as their relatively narrow trochlea with pronounced anteromedial and posterolateral lips, and relatively short, posteriorly-directed medial epicondyle are absent among the Fayum apes and parapathecids and represent derived conditions for cercopithecoids.

Thus, the new fossil evidence indicates that relative to the earliest known catarrhines, both modern apes and modern cercopithecoids show derived morphologies in the elbow region. To characterise early fossil hominoids as 'monkey-like' or 'ape-like' is to pose a false dichotomy. Rather, what can be seen in the humeral anatomy of the Fayum hominoids (as well as in many other aspects of the skeleton and cranium of these animals) is a distinct and more primitive morphology comparable to that found among the third group of living higher primates, the New World monkeys. Functionally, both the ulna^{1,3,10} and humerus indicate that *Aegyptopithecus* was a largely quadrupedal, arboreal animal and that the best models for locomotor behaviour of early hominoids are neither the extant apes nor the extant cercopithecoid monkeys. Instead, closest resemblances are with the larger arboreal quadrupeds among platyrrhines, in particular the howler monkey *Alouatta*, which seems to demonstrate today a forelimb morphology similar to that exhibited by Oligocene apes.

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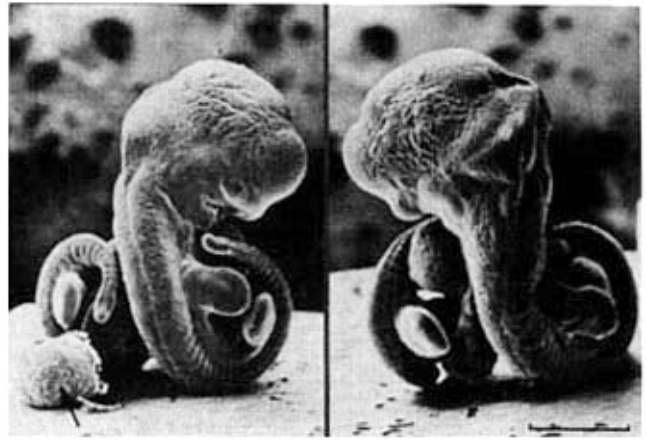


Fig. 1 Scanning electron micrographs showing two views of conjoined twin isolated on the twelfth day from a female injected with vincristine sulphate on the seventh day of gestation. A single head and thorax and two complete vertebral columns may be seen. A small round mass of tissue (arrowed) was attached to the umbilical region of one of the fetuses by a cord containing blood vessels. Scale bar, 1 mm.

report that a low incidence of gross anomalies was observed in fetuses on days 10 and 12 of gestation when pregnant females were treated with a single dose of this agent on the sixth, seventh or eighth day of gestation. However, contrary to expectation, a high proportion of litters from females treated on the seventh and eighth day of gestation contained at least one set of monozygotic twins. In this respect the present study is unique in that an experimental method of inducing a low but significant incidence of identical twinning in a mammal is reported. Furthermore, this work provides direct evidence regarding the comparatively late stage of embryonic development when identical twin formation may be induced in the mouse, as the most advanced stage at which twinning could be induced with vincristine was at the early headfold stage. However, the highest incidence of monozygotic twinning was obtained when pregnant females were treated on the morning of the seventh day of gestation, when embryos would be expected to be at the advanced egg-cylinder stage. It was in this latter group that a single conjoined twin of the janiceps type (cephalothoracopagus) was obtained. The possible aetiology of monozygotic and conjoined twin formation is briefly discussed here in the light of these experimental findings.

Female mice, 6-8-week old virgin CFLP (Anglia), were mated with males of the same strain, and isolated on the morning of finding a vaginal plug (designated the first day of pregnancy). Females were injected intraperitoneally with 0.3 mg per kg vincristine sulphate in 0.2 ml normal saline on the morning of the sixth, seventh or eighth day of gestation. Controls were injected with a similar volume of normal saline. Autopsies were carried out on either the tenth or twelfth day of gestation. The uterine contents were examined, and the number and location of resorption sites and normal and abnormal fetuses recorded (Table 1).

In the only anomalous fetuses encountered, the region overlying the fourth ventricle was apparently collapsed, giving this region of the hindbrain an abnormally flattened appearance. These fetuses were otherwise apparently quite normal and healthy at the time of autopsy. It is not immediately clear whether the neural tissue in the hindbrain region of this comparatively small population of fetuses would have developed into, for example, an encephalocele which would have been recognisable at a later stage of gestation. It is also unclear whether these fetuses would have survived to term or into the early post-natal period. A higher incidence of fetuses with abnormal heads of this type was observed in the population of fetuses isolated from females treated on the seventh day (6.2%), compared with those isolated from females treated on

Induction of monozygotic twinning in the mouse

VINCRIStINE SULPHATE (Oncovin, Lilly) is a drug commonly used in the chemotherapy of acute leukaemias in children, lymphomas and certain other malignant conditions. We describe here experiments investigating the teratogenicity of this drug during the early stages of embryogenesis in the mouse. We

Table 1 Incidence of resorptions, normal and abnormal fetuses and monozygotic twins in female mice treated with vincristine

Treatment	Day of injection	Day of autopsy	Total females examined	Implantation sites		Abnormal heads	Sets of twins
				Living fetuses	Resorptions		
Vincristine	6	10	3	39	1	1	0
	7	10	5*	64	1	5	3
	7	12	4	48	3	2	1
							(conjoined)
	8	10	4	52	1	1	1
	8	12	2	25	1	1	0
Saline	6	10	3	37	1	0	0
	7	10	4	51	2	0	0
	8	10	3	38	0	0	0

Female mice were injected with vincristine sulphate or saline (controls) on the sixth, seventh or eighth day of gestation.

* An additional two litters contained only resorbed embryos.

the sixth (2.6%) and eighth (2.6%) day of gestation. This low incidence of easily recognisable neural tube pathology is of interest in the light of earlier observations of Joneja and Ungthavorn¹, who investigated the effect of an intraperitoneal injection of this agent in mice on the ninth day of pregnancy. In addition to an increase in the incidence of resorptions, these workers observed that out of 77 fetuses examined at term 5.2%, 11.7% and 6.5%, respectively, were either microcephalic, exencephalic, or had an encephalocele. This difference may be related to the strain of mice used, or more probably to the later time of administration of this teratogen. Similar central nervous system anomalies have also been reported when hamsters were injected with this agent on the eighth day of gestation².

Another observation that seemed to be related to the time of exposure of pregnant females to this agent in the present series was the incidence of monozygotic twins. Four sets of twins, one pair of which was conjoined, were observed when females were treated on the seventh day of pregnancy. Of the five females autopsied on the tenth day, three each had one set of twins, and a single pair of conjoined twins (Fig. 1) was isolated from one out of four females autopsied on the twelfth day of gestation. One additional set of twins was obtained from a female treated on the eighth day. At least three of the twins shared a common amniotic cavity. No twins were observed in the fetuses isolated from females treated on the sixth day of gestation. Apart from the conjoined twins all the other twin fetuses seemed to be morphologically normal.

The main interest in the present study lies in the experimental production of monozygotic twinning in the mouse, as the normal incidence in this and most other mammalian species is thought to be extremely low^{3,4}. In fact, polyembryony is extremely rare and only thought to occur regularly in certain species of armadillo⁵. It is generally thought that monozygotic twins probably result from the fission of a single embryonic axis shortly after the blastocyst stage has been reached, most probably during the early post-implantation period^{6,7}. It is therefore interesting that the period of embryogenesis that was most susceptible to the twinning stimulus provided by vincristine in the present study was from the advanced egg-cylinder stage to the early headfold stage of development, and it is at this latter stage that mouse embryos have a distinct head process and primitive streak⁸. Rarely, incomplete separation of these embryonic axes occurs, and results in the formation of conjoined twins. This seems the simplest explanation for the isolated examples of conjoined twins reported in this and in earlier studies in which pregnant hamsters^{4,9}, rabbits¹⁰ and rats¹¹ were treated with teratogens, and as spontaneous occurrences in other species.

The remarkably few reports in the literature in which monozygotic and conjoined twins have been recorded as spontaneous events and following treatment of pregnant animals with teratogens seems to indicate that twins of these types were previously encountered only as isolated occurrences, unlike the present study, where they appeared to be a definite response to the vincristine treatment.

The present experimental approach thus allows for the first time detailed examination of the early stages in the development of monozygotic twins, at precise intervals after the twinning stimulus has been given. Although the exact nature of the twinning stimulus evoked by vincristine remains to be established, it should now be possible to investigate monozygotic twinning in relatively controlled conditions.

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Plasmid-induced loss of virulence in *Vibrio cholerae*

WE have previously reported that pathogenicity of *Vibrio cholerae* was suppressed by introducing P or V plasmids and that this effect was due to decreased toxin production¹. This work has been extended to show that cells harbouring both P and V plasmids become attenuated and do not cause experimental cholera. Such attenuated strains are attractive candidates for live oral vaccines if it can be shown that the immunogenicity of plasmid-harboring strains is not altered and that they remain stable. We provide here convincing evidence that the cells are not cured of their plasmids *in vitro* or *in vivo* and do not lose their immunogenic potential.

Strain KB9 served as the parent virulent strain. P and V plasmids were introduced in KB9; two strains, designated as KB9:PV and CD24, were isolated, apparently different in one respect—that P is not expressed in CD24. This is not surprising because P is often repressed by V (ref. 2). Strain 569B is a comparatively poor pathogen *in vivo* but produces significant amounts of toxin *in vitro*. CD23 was derived from 569B and

characterised as having P and V plasmids. KB92 (Ogawa) and KB93 (Inaba) were virulent challenge strains. The properties of strain KB9, and the technique of introducing P and V plasmids, characterisation of plasmids, growth of vibrios, media and buffer have been described in our previous report¹.

The pathogenicity of vibrio strains was examined in the ileal loop of adult rabbits³ and intra-intestinal infection of vibrios in infant rabbits⁴. $1-2 \times 10^9$ cells of KB9:PV and CD24 in 1 ml brain-heart infusion broth (Difco) were injected separately into the ileal loops of rabbits and the open intestine of infant rabbits. Neither diarrhoea nor death was recorded in the infant rabbits up to 48 h and ileal loops of the adult rabbits were without any accumulation of diarrhoeal fluid. KB9, on the other hand, caused death of the infant rabbits with 10^5-10^6 organisms and ileal loops were full of fluid. For each strain, eight infant rabbits and an equal number of adult rabbits were used. LD₅₀ values of these strains were estimated in Swiss mice¹ and found to be 9×10^6 and 10^7 organisms for KB9, KB9:PV and CD24, respectively. These data suggest that plasmid-bearing cells of *V. cholerae* behave as attenuated strains.

As 569B released significant amounts of toxin in Syncase minimal medium¹, CD23 was grown in similar conditions. Assay of the toxin in the culture filtrate of CD23 gave negative results. This is consistent with our earlier observation¹ that P and V plasmids interfered with the synthesis or release of toxin.

PV strains underwent two successive passages in rabbit intestine. The loss of plasmids was never detected. These strains were subcultured several times in broth, exposed to acriflavine and sodium dodecyl sulphate, and maintained at 37 °C in broth for several weeks. Individual colonies were tested but none were found to have lost plasmids. A large number of agents which cure *Escherichia coli* of plasmids could not eliminate the sex factor P in *V. cholerae*⁵. In addition, we have evidence that when plasmids are transferred from one cell to another in conjugal mating, a copy is always left behind in the donor. Unlike *E. coli*⁶, P⁺ or V⁺ donors remain P⁺ and V⁺ after mating. The experiment used was as follows: the donor was P⁺, Str^s, and the recipient was a Str^R, ile⁻ val⁻ arg⁻ his⁻ strain. After bacterial mating⁷, the mixture was plated on minimal glucose salt medium to score the donor population, and on media supplemented with amino acids and streptomycin to score the recipient. Individual colonies were tested and it was found that all the colonies of the donor strain were P⁺ and among the recipients >60% were P⁺. A similar result was obtained with a V⁺ donor; however, the efficiency of transfer of the V plasmid was very low. These results strongly suggest that plasmids are very stable in *V. cholerae*.

Immunogenicity was examined by immunising rabbits subcutaneously with KB9:PV and CD24. Table 1 indicates a good correlation between protection to challenge and vibriocidal antibodies in the serum. Both strains conferred good protection

in Pittman and Feeley's mouse protection test⁸, particularly against homologous challenge strains.

Thus, we propose that plasmid-bearing strains like KB9:PV and CD24 may be used as live oral vaccines. These strains possess several other features of a vaccine strain⁹ and these will be reported elsewhere.

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Accessory cell requirement for anti-IgM-induced proliferation of B lymphocytes

THE nature of the signals which B lymphocytes must receive so as to proliferate and differentiate are not yet completely understood. It is likely that B cells with different triggering requirements exist^{1,2}, and that B-cell proliferation and B-cell differentiation require separate signals³⁻⁶. Because of this heterogeneity in signalling, it has been difficult to define precisely what is necessary to induce the expansion and differentiation of a particular B-cell clone after its exposure to antigen. It has been suggested that direct antigen binding to specific membrane immunoglobulin initiates at least one of the necessary activation signals⁷⁻⁹. Further activation signals may come from T cells and/or macrophages⁷⁻⁹. We have been studying the nature of the stimuli required for the induction of B-cell proliferation. We report here that using soluble goat anti-mouse IgM as a surface Ig cross-linking agent, we have confirmed that interactions with surface Ig can induce the proliferation of at least a subset of mouse B cells¹⁰⁻¹². In addition, we have found a requirement for radioresistant phagocytic adherent cells in the polyclonal stimulation of B cells by this antibody.

Figure 1 shows the goat anti-IgM-induced proliferation of CBA/Tufts spleen cells. Optimal stimulation of 5×10^5 lymphocytes was consistently seen with 1-5 µg of MOPC-104E affinity column-purified goat anti-MOPC-104E (µ,λ) and TEPC-183 (µ,κ). Although 10-50 µg of normal goat Ig per culture induced a slight amount of proliferation, the stimulation was less than twofold above background. This is in contrast to the 13-fold stimulation seen with 1.25 µg of goat anti-IgM.

Purified µ,λ (MOPC-104E) or µ,κ (TEPC-183) myeloma proteins were equally effective in abrogating the proliferative response induced by the optimal dose of goat anti-IgM¹³. As little as 0.5 µg per culture of MOPC-104E or TEPC-183 completely inhibited anti-IgM-induced, but not lipopolysaccharide (LPS)-induced proliferation. Up to 4 µg per culture of

Table 1 Protective immunity and serum vibriocidal antibody titre in rabbits immunised with KB9:PV and CD24

Vaccine	Challenge strains	% Immunity to challenge	Vibriocidal titre (mean)
KB9:PV	KB92	74	3×10^5
	KB93	100	9×10^4
CD24	KB92	81	1×10^5
	KB93	77	8×10^4
Control	KB92	0	<50
	KB93	0	<50

Swiss albino rabbits weighting 1-1.2 kg were used at the time of immunisation. KB9:PV and CD24 were grown in brain-heart infusion broth and 2×1 ml of live culture ($1-2 \times 10^9$ ml⁻¹) was administered subcutaneously to each rabbit at 21-d intervals. Groups of 8-10 rabbits were immunised. They were challenged on the 35th day after the second dose of vaccine and starved for 48 h before challenge. Immunity to challenge was assessed in ligated ileal loops³ and expressed as reported previously¹⁰. Serum vibriocidal titres were determined according to Finkelstein¹¹ on serum samples collected 1 d before challenge.

Table 1 ^3H -Thymidine incorporation expressed as $\bar{X} \Delta$ c.p.m. $\pm$ s.d. (stimulation index)

	2 μg goat anti-IgM		0.5 μg K-235 LPS		0.25 μg Con A	
Expt 1						
Normal spleen cells	112,632 $\pm$ 22,934	(12)	124,464 $\pm$ 6,788	(13)	418,281 $\pm$ 8,476	(40)
M-depleted spleen cells	5,500 $\pm$ 1,982	(3)	126,099 $\pm$ 1,936	(40)	306,206 $\pm$ 4,860	(96)
M-depleted spleen cells + normal peritoneal M ϕ s	124,864 $\pm$ 4,961	(10)	89,697 $\pm$ 7,829	(7)	403,908 $\pm$ 4,084	(29)
Expt 2						
Normal spleen cells	127,299 $\pm$ 14,512	(12)	228,083 $\pm$ 5,789	(21)	503,516 $\pm$ 18,623	(45)
Spleen cells + 50 μg silica	34,606 $\pm$ 13,641	(6)	237,780 $\pm$ 2,227	(38)	436,632 $\pm$ 14,725	(69)

In expt 1, normal CBA/Tufts spleen cells were depleted of macrophages (M) by 2-h adherence to plastic Petri dishes in the presence of 20% fetal calf serum (FCS) and 2 subsequent passages of nonadherent cells over Sephadex G10 columns¹⁵ prewashed with warm RPMI-1640 + 20% FCS. Macrophage-depleted cells contained less than 1% neutral red-positive, latex-positive cells. Adherent normal peritoneal cells were obtained by peritoneal lavage and 3-h adherence to plastic Petri dishes. Adherent cells removed in 1:5,000 EDTA-phosphate-buffered saline with a rubber policeman were 70% neutral red-positive, latex-positive. Adherent cells were irradiated with 1,200 rads. 5×10^3 Macrophages were added to cultures of 5×10^5 macrophage-depleted cells. A peak stimulatory dose of 2.5 μg goat anti-IgM, a dose of 0.5 μg *Escherichia coli* K235 LPS, a dose of 0.25 μg Con A or medium only was added to each culture. ^3H -thymidine incorporation is expressed as Δ = (experimental minus control) and as stimulation index = (experimental/control). In expt 2, normal spleen cells were incubated with 2.5 μg goat anti-IgM, 0.5 μg K235 LPS, 0.25 μg Con A, or medium. 50 μg silica (particle diameter $< 5 \mu\text{m}$) was added to one series of cultures.

purified γ, κ (MOPC-21) myeloma protein failed to affect the response¹³. Hence, the proliferative response induced by the affinity-purified antibody was apparently due to anti- μ activity and not to any anti- λ or anti-idiotypic activity in the preparation.

The spleen cell proliferation induced by anti-IgM seems to be a T-independent phenomenon. Spleen cells from nude mice and spleen cells depleted of T cells by T cell-specific cytotoxic antisera + complement show intact anti-IgM-induced proliferative responses¹⁴ (our unpublished results).

We investigated the requirement for accessory macrophages in this B-cell activation system. Adherent cells were removed from preparations of nucleated spleen cells by 2-h adherence to plastic and two sequential passages through Sephadex G10 columns. Such depleted cells were poor responders to goat anti-IgM (Table 1, expt 1). The addition of irradiated adherent

cells from normal peritoneum restored the B-cell response to that seen with normal spleen cell suspensions.

Further evidence that macrophages are ancillary cells for anti-IgM-induced proliferation was obtained from experiments in which silica, a selective toxic agent for phagocytic cells¹⁶, was added to cultures. Silica, 50 μg , significantly depressed the spleen cell response to anti-IgM without affecting the response to LPS, another polyclonal B-cell activator (Table 1, expt 2).

The response to LPS was also unaffected by adherent cell depletion (Table 1, expt 1). In fact, the addition of adherent peritoneal cells to depleted cell cultures suppressed the LPS response. These characteristics of B-cell LPS responses have been previously noted^{17,18}.

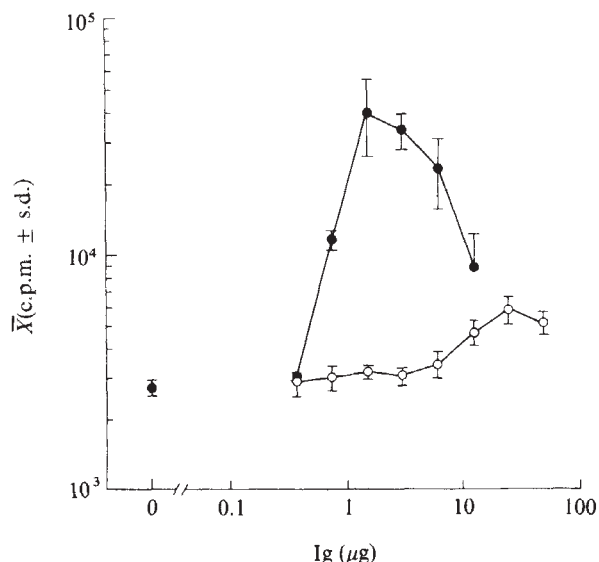
Unlike the response to LPS, the B-cell proliferative response to the mitogen dextran sulphate has been shown to be macrophage-dependent¹⁷. Whether the apparent macrophage independence of the LPS response, in contrast to the dextran sulphate and anti-IgM responses, is a consequence of the unique activating properties of the LPS molecule^{19,20} or a reflection of subsets of B cells with different macrophage requirements for activation is not yet known.

Our macrophage depletion techniques failed to decrease the macrophage-dependent T-cell response to concanavalin A (Con A)²¹ to the same extent as the B-cell response to anti-IgM (Table 1). This differential sensitivity to macrophage depletion may reflect differences in the amount of macrophage help required for the stimulation of diverse lymphocyte populations or may reflect a heterogeneity in ancillary macrophage populations^{22,23}.

Our results suggest that, in the presence of macrophages, binding of molecules to surface IgM can activate certain B cells to proliferate. The precise role of the macrophage in this system is not yet clear. Macrophages may contribute secondary signals needed to push B cells further into the cell cycle. Alternatively, they may promote the viability of the B-cell population stimulated by anti-IgM, or they may present anti-IgM to B cells in an insoluble array. We feel the latter possibility is unlikely because: (1) anti-IgM-pulsed and washed macrophages are unable to stimulate macrophage-depleted lymphocytes in the absence of re-added soluble anti-IgM (unpublished); (2) Sieckmann *et al.* have shown that Fab₂, which is incapable of Fc-receptor binding to macrophages²⁴, and whole Ig preparations of goat anti-IgM are equally stimulatory for mouse cells¹⁰; and (3) 2-mercaptoethanol added to cultures can overcome the effects of macrophage depletion¹³. Whether 2-mercaptoethanol potentiates the effect of residual macrophages in adherent cell-depleted cultures or substitutes for macrophage function in the stimulation of anti-IgM responsive B cells is not known.

The population of B cells which responds to anti-IgM is thus far analogous to the population which responds to most thymus-independent antigens and the population which produces colonies in *in vitro* soft agar cultures. All these functional

Fig. 1 Stimulation of CBA/Tufts spleen cells with goat anti-IgM. Various concentrations of MOPC-104E column-purified goat anti-(MOPC-104E, TEPC-183 (●) or normal goat Ig (○) were added to 5×10^5 spleen cells in triplicate flat-bottomed micro-culture wells to give a total volume in each well of 200 μl . Cultures were maintained in RPMI-1640 + 5% heat-inactivated FCS, with 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 20 mM HEPES (Sigma) and 2 mM glutamine. After 48 h of culture at 37°C in a humidified, 5% CO₂ incubator, 1 μCi of [methyl- ^3H]thymidine (6.7 Ci mmol⁻¹, NEN) was added to each culture. After 24 h, cells were collected and the ^3H -thymidine incorporation was measured. The means of triplicate cultures $\pm$ standard deviations were calculated for experimental and control cultures.



populations have been found to be reduced or absent in CBA/N mice and to require macrophages, but not T cells, for their activation²⁵⁻²⁸.

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Occurrence of Ia antigens on tissues of non-lymphoid origin

UNLIKE the classical transplantation antigens, called HLA-A,B,C in man and H-2K,D in mouse, the HLA-DR (Ia) antigens have a restricted tissue distribution¹. Primarily cells belonging to the immune system express Ia antigens whose only known biological role is to participate in cell-to-cell cooperation events involving lymphocytes and macrophages²⁻⁴. However, cells of the immune system do not only interact among themselves but they also associate physically with other types of cells like epithelial cells of the intestine and the lactating mammary gland⁵ and with reticuloepithelial cells of the thymus⁶. The possibility that all such interactions are under Ia antigen control is raised by indirect immunofluorescence analyses which show that Ia antigens are expressed on a great variety of epithelia. We report here our examination of several cryostat sections of guinea pig tissues for the presence of Ia antigens by indirect immunofluorescence. To obtain staining patterns that were reproducible and significantly different from the controls it proved necessary to use immunosorbent-purified heteroantibodies (see legend to Fig. 1 for description of the antibodies).

The specificity of the isolated antibodies was tested in indirect immunoprecipitations⁷ of radioactive cell membrane glycoproteins⁸. SDS-polyacrylamide gel electrophoresis⁹ of precipitated

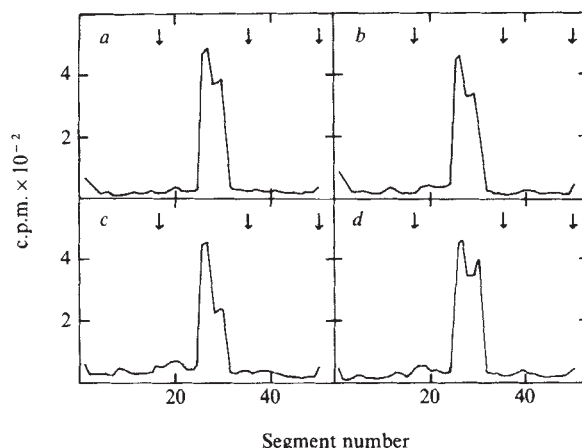


Fig. 1 SDS-polyacrylamide gel electrophoresis of ³H-labelled cell surface glycoproteins indirectly immunoprecipitated with immunosorbent purified antibodies raised against HLA-DR antigens. Single cell suspensions were prepared from spleen (a), small intestine²⁰ (b), and parotid gland²¹ (c), and Kupffer cells were isolated from the liver²² (d) of outbred guinea pigs. The cells were surface labelled with ³H (ref. 8), membrane proteins were solubilised with Triton X-100, glycoproteins were isolated by lectin affinity chromatography and indirect immunoprecipitations were carried out. Control precipitates were generated with normal rabbit serum. Precipitated molecules were subjected to SDS-polyacrylamide gel electrophoresis. The control precipitates did not contain significant amounts of radioactivity (not shown). The immunosorbent-purified antibodies were derived from a rabbit antiserum against highly purified HLA-DR antigens. The reactivity of this antiserum has been documented in detail elsewhere¹⁴ and it has been shown that this antiserum crossreacts extensively with guinea pig Ia antigens²³. The antiserum was passed over a Sepharose-4B column containing highly purified, covalently bound HLA-DR antigens isolated from chronic lymphatic leukaemia cells (L.K., L.T., L.R. and P.A.P., submitted). The bound antibodies were desorbed with 0.2 M sodium citrate buffer, pH 2.9. After extensive dialysis against phosphate-buffered saline (PBS), pH 7.2, the antibodies were concentrated. The arrows (left to right) denote the migration positions of marker IgG heavy and light chains and the tracking dye bromphenolblue.

molecules derived from guinea pig splenocytes, intestine epithelial cells, salivary gland epithelial cells and Kupffer cells revealed the occurrence of Ia antigen-like proteins¹⁰ on all four cell types (Fig. 1). The high specificity of the antibodies was ascertained by the absence on the gels of material distinct from the Ia-antigen subunits.

The results of the indirect immunofluorescence studies with use of the specific antibodies on cryostat sections from various tissues are summarised in Table I. In all tissues examined only epithelial cells were stained. The exception to this was the liver where only the Kupffer cells reacted convincingly with the antibodies whereas the hepatocytes appeared devoid of Ia antigens. The earlier reports that the Langerhan's cells of epidermis express Ia antigens^{11,12} were confirmed. The cornea contains similar types of cells as the antibodies bound specifically to cells displaying short dendritic processes. A strong, patchy fluorescence was noted along the border of all intestinal sections studied (Fig. 2a). The cells lining the bronchi in the respiratory tract were generally stained rather diffusely. The epithelia of the gall bladder and the kidney pelvis (Fig. 2b) were well stained in some parts and weakly in others. The urinary bladder epithelium also reacted well with the antibodies inasmuch as a strong fluorescence was observed, particularly in the crypts. The mammary gland from virgin animals contained few if any cells that were stained by the anti-HLA-DR antigen antibodies. However, in sections of lactating mammary glands an intensive fluorescence was noted around the epithelial cells lining the ducts (Fig. 2c). These cells produce the milk fat droplets which are enveloped by plasma membrane from the epithelial cells¹³. Figure 2d shows that also the membranes of

the milk fat globules displayed a bright fluorescence after staining with the antibodies.

Antibodies gave a discrete but uniform fluorescence mainly on rather large cells when sections of thymus were examined. The antibodies do not react with thymocytes¹⁴ so it seemed that the stained cells probably belonged to the reticuloepithelium.

The relative amounts of the anti-HLA-DR (Ia) antigen antibodies bound to single cells obtained from the small intestine and spleen were compared. Figure 3 shows that those spleen cells that bound the antibodies contained about threefold more

Fig. 2 Indirect immunofluorescence of thin sections of small intestine (a), kidney pelvis (b), lactating mammary gland (c) and milk fat globule membranes (d) of guinea pigs after staining with immunosorbent-purified anti-HLA-DR antigens antibodies (see legend to Fig. 1). The tissues were immediately frozen in cold isopentane (-70°C) and sections of $6\text{ }\mu\text{m}$ were subsequently prepared. The air-dried sections were incubated with $50\text{-}\mu\text{l}$ portions of the antibodies and normal rabbit IgG, respectively. The IgG-fractions, used at a concentration of about $10\text{ }\mu\text{g ml}^{-1}$, were diluted in PBS, pH 7.2, containing 4% bovine serum albumin (BSA). The antibodies were allowed to react with the sections for 30 min at room temperature in a humidified atmosphere. The sections were then washed extensively in PBS-BSA and allowed to react with fluorescein-isothiocyanate-conjugated swine-anti-rabbit IgG antibodies (Dakopatts) at a concentration of 0.5 mg ml^{-1} . After an incubation period of 30 min and several washes in PBS-BSA the reactions were mounted in 10% glycerol-PBS, pH 7.2. The sections were examined in a Zeiss fluorescence microscope with incident light and blue narrow band activation (chromatic mirror 510 and interference filters KP 490 and KP 500 and orange filter LP 520) illuminated with an Osram HBO 200 lamp. Sections stained with normal rabbit IgG did not display any fluorescence (not shown).

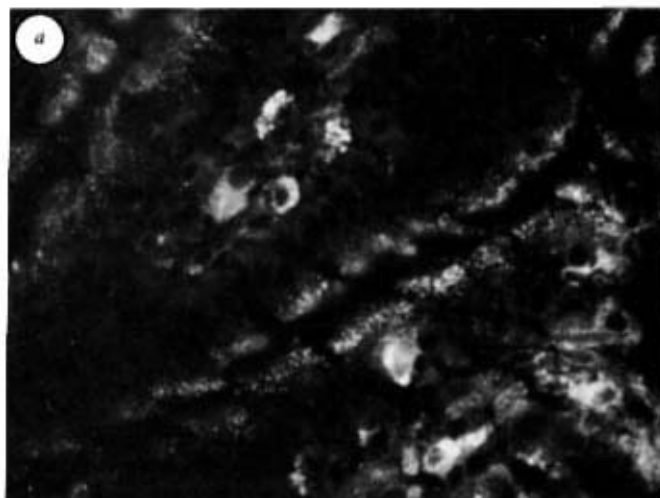


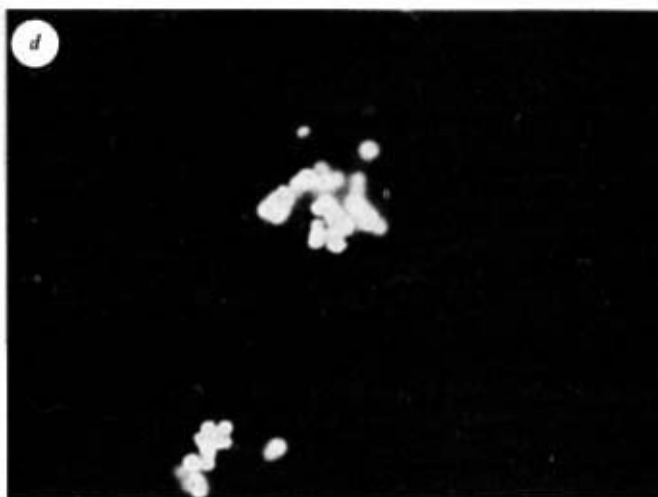
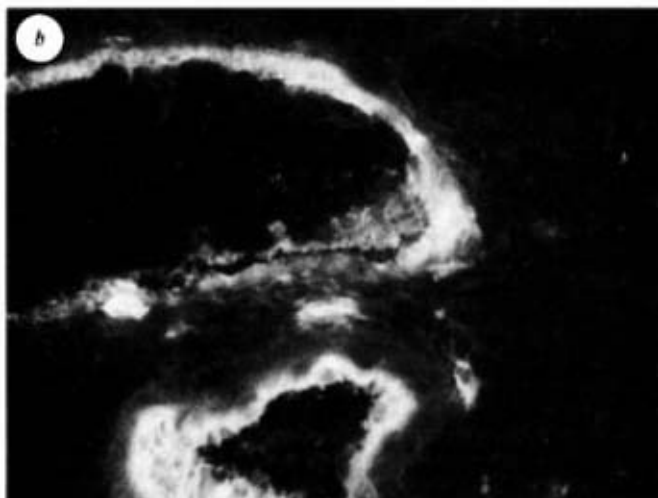
Table 1 Occurrence of Ia-antigen-like molecules on cells of various organs*

Tissue	Cell type stained†	Presence of Ia antigen subunits‡
Salivary glands (parotid and submandibular glands)	Glandular epithelium	+
Oesophagus (upper part)	Not detected	nt
Small intestine (duodenum; jejunum; ileum)	Epithelial cells	+
Colon	Epithelial cells	nt
Liver	Kupffer cells	+
Gall bladder	Epithelial cells	nt
Cornea	Langerhans-like cells	nt
Epidermis	Langerhans' cells	+
Mammary gland (virgin)	Few Ia-antigen expressing cells	nt
(lactating)	Epithelial cells	+
Trachea	Not detected	nt
Bronchi	Epithelial cells	nt
Urinary bladder	Epithelial cells	nt
Kidney pelvis	Epithelial cells	nt

* All analyses were performed on tissues from guinea pigs. Ia-antigen-like molecules were identified with use of immunosorbent-purified anti-HLA-DR antigen antibodies.

† Determined by indirect immunofluorescence on thin cryostat sections.

‡ Determined by indirect immunoprecipitation and SDS-poly-acrylamide gel electrophoresis of ^3H -labelled cell surface glycoconjugates. nt, not tested.



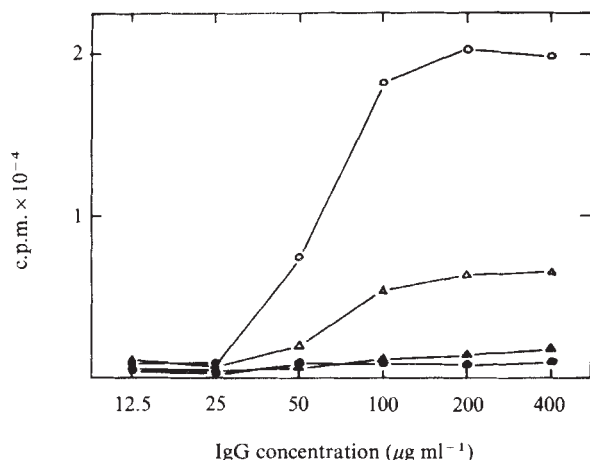


Fig. 3 Binding of immunosorbent-purified anti-HLA-DR antigen antibodies to guinea pig spleen cells (O, ●) and small intestine epithelial cells (Δ, ▲). Aliquots containing 5×10^5 spleen cells and 2×10^5 epithelial cells²⁰, respectively, were incubated with various amounts of immunosorbent purified antibodies (O, Δ) and normal rabbit IgG (●, ▲), respectively, in a final volume of 0.1 ml of PBS-BSA. After an incubation period of 30 min at +22 °C the cells were washed extensively and suspended in 0.05 ml of PBS-BSA containing saturating amounts of ¹²⁵I-labelled protein A (ref. 24). After a further incubation period of 30 min at 22 °C the cells were repeatedly washed and subjected to radioactivity measurement. Indirect immunofluorescence of the two single cell suspensions demonstrated that all of the epithelial cells but only about 40% of the spleen cells reacted with the antibodies. The number of spleen cells used in the binding experiment was adjusted accordingly.

of the radioactivity than did the intestine epithelial cells. As the latter type of cells are considerably larger than the lymphocytes the density of the Ia antigens per unit surface area on the epithelial cells must be only a fraction of that of the B lymphocytes.

This study was undertaken with use of immunosorbent-purified heteroantibodies. The occurrence on various tissues of molecules reactive with the antibodies does not necessarily imply that true Ia antigens were recognised inasmuch as the heteroantibodies may have cross-reacted with other components. This possibility seems unlikely, however, as the only cell surface-labelled glycoconjugates that reacted with the antibodies displayed the typical Ia antigen subunit pattern on SDS-polyacrylamide gel electrophoresis. Moreover, highly purified intestinal epithelial cells of mice will give rise to Ia antigen alloantibodies when injected into I-region congenic mice. Such alloantisera react with Ia antigens expressed on splenocytes. Some conventional anti-Ia antigen alloantisera also react specifically with the intestinal cells (B.C., L.R. and P.A.P., unpublished observations). Whether the Ia antigens present on cells of non-lymphoid origin belong to any particular sublocus of the MHC I-region remains to be elucidated.

The demonstration of Ia antigens on epithelial cells in various tissues has bearing upon clinical organ transplantations. The presence of Ia antigens on kidney cells is in keeping with the observation that HLA-DR antigen identity between the donor and the recipient is of as great importance as HLA-A,B,C antigen identity¹⁵.

The association between some types of diseases and the HLA-DR antigen phenotype¹⁶ also has bearing on the present findings. Thus, the expression of HLA-DR antigens of a given phenotype on cells of the disease-prone organs might represent an absolute requirement for manifestation of the disease.

It is interesting that Ia antigens are expressed on reticulo-epithelial cells of the thymus in view of the recent observations that the education of T cells in the thymus is dependent on the I-region genotype of the reticuloepithelial cells¹⁷. It is tempting to suggest that it is the Ia antigens on the reticuloepithelial cells that are crucial for the functional maturation of the T cells. The

abundance of IgA-secreting cells in various epithelia¹⁸ may be related to the occurrence of Ia antigens in these tissues. This is particularly important for the mammary gland. In virgin animals very few IgA-secreting plasma cells are found in the gland but under the influence of lactotropic hormones IgA-secreting cells accumulate in the epithelium of the gland¹⁹. This sequence of events is strictly parallel to the expression of Ia antigens on the epithelial cells (L.K., U.F. and P.A.P., unpublished observations).

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Localisation of Chido and Rodgers determinants to the C4d fragment of human C4

CHIDO (Ch) AND RODGERS (Rg) are blood-group antigens present in plasma and on red cells. Interest in these antigens was stimulated when Ch (ref. 1) and Rg (ref. 2) were found to be genetic markers for the major histocompatibility complex, HLA, and when strong linkage disequilibrium between the Rg-negative phenotype and HLA-A1, B8 homozygosity was noted³. Ch and Rg recently attracted a broad audience when O'Neill *et al.*⁴ showed that the Ch and Rg phenotype of plasma was correlated with the electrophoretic polymorphism of human C4, the fourth component of complement, and that C4 purified

Table 1 Agglutinin titres of anti-C4, anti-C3, anti-Ch and anti-Rg with red cells coated with complement

Source of complement	Ch, Rg phenotype	LIS % sucrose	Anti-C4c	Anti-C4d	Antiserum Anti-C3b	Anti-C3d	Anti-Ch	Anti-Rg
EDTA plasma	Ch+, Rg+	10	0	0	0	0	0	0
ACD plasma	Ch+, Rg+	10	>10,000	>1,600	>500	>120	>2,000	>2,000
Serum	Ch+, Rg+	10	128,000	>1,600	>500	>120	2,560	2,560
	Ch+, Rg+	6	64,000				2,560	1,280
Serum	Ch-, Rg+	10	32,000	>1,600	>500	>120	0*	2,560
	Ch-, Rg+	6	500				0	0
Serum	Ch+, Rg-	10	64,000	>1,600	>500	>120	1,280	0*
	Ch+, Rg-	6	100				0	0
C2D serum	Ch+, Rg+	10	>10,000	>200	0	0	>400	>400
C4D serum	Ch-, Rg-	10	0		0	0	0	0
C4D serum	Ch-, Rg-	10	0		0	0	0	0
Serum	Ch+, Rg+	9.24	0	0	>500	120	0	0
	Ch-, Rg+	9.24	0	0	>500	100	0	0
	Ch+, Rg-	9.24	0	0	>500	50	0	0
Anti-Le ^a serum	Ch+, Rg+	none	>1,000	200			>8	>8

As a source of complement, serum, ACD plasma and EDTA plasma was obtained from donors of known Ch and Rg phenotype. Red cells were coated with complement by activation in low-ionic-strength solution (LIS): 10% aqueous sucrose⁸; 6% sucrose/0.15% NaCl (ref. 12); 9.24% buffered sucrose containing EDTA, purported to coat cells with C3 without detectable C4 (ref. 11). C4D serum was from a C4-deficient patient; C2D serum was from a C2-deficient patient. Immune transfer of complement to red cells was effected using fresh serum containing anti-Le. Red cells were tested with anti-complement reagents diluted in 22% bovine serum albumin (Ortho Diagnostics). Rabbit anti-human C4 (Behringwerke AG) reacted strongly with red cells coated with C4b but not with these cells after trypsin treatment, or with red cells sensitised *in vivo* with C4/C3; this serum is referred to as anti-C4c. Anti-C4d (two examples) was raised in monkeys injected intravenously with autologous red cells coated with C4d only; these sera were non-precipitating⁵. Anti-C3b (Behringwerke) was obtained as anti-human C3. Anti-C4c, anti-C4d, and anti-C3b were used unabsorbed at dilutions of 1:50 or greater. Anti-C3d was absorbed with human red cells. Six examples of anti-Ch and five examples of anti-Rg from patients immunised by transfusion or pregnancy were used.

* Weak and variable background reactions were observed at all dilutions of antiserum; these reactions were abolished by trypsin-treatment of the red cells and were probably due to immune adherence.

to homogeneity inhibited anti-Ch and anti-Rg. We have studied the transfer of Ch and Rg from plasma to red cells in conditions in which C4 is transferred. We report here a correlation between the Ch, Rg polymorphism and functional C4, and show that Ch and Rg reside on the C4d fragment of the molecule. We also report that a monkey antiserum to human C4d (ref. 5) is predominantly anti-Ch and anti-Rg.

The antibodies anti-Ch and anti-Rg have been described as 'nebulous'⁶ and 'serologically difficult'⁷ because of their weak and variable reactions with red cells; Ch and Rg can usually be detected only by the antiglobulin test. We found, however, that Ch and Rg could be readily detected by direct agglutination using anti-Ch and anti-Rg when the antigens were transferred to red cells *in vitro* by activation of the classical complement pathway in low-ionic-strength solution (LIS). In 10% sucrose LIS⁸, red cells became strongly Ch(Rg)-positive when serum or ACD plasma was used as a source of C4, but not when EDTA plasma was used; the Ch(Rg) phenotype acquired by the red cells was entirely dependent on the Ch(Rg) phenotype of the donor serum (Table 1). Red cells acquired both Ch and Rg from C2-deficient serum, but neither Ch nor Rg from C4-deficient serum; C4-deficient serum did not inhibit anti-Ch or anti-Rg. Heating serum to 56 °C for 60 min (ref. 9), or treatment with thiocyanate¹⁰ which inactivates C4, C3 and C5, abolished the transfer of Ch(Rg) to red cells in LIS. Using a LIS technique¹¹ which coats red cells with C3b without C4, the cells did not acquire Ch(Rg). Immune transfer of Ch(Rg) was demonstrated by exposing Le(a+) red cells to fresh serum containing complement-binding anti-Le^a (Table 1).

We found that Ch+, Rg- and Ch-, Rg+ serum could be readily distinguished from Ch+, Rg+ serum by the amount of C4 transferred to red cells in 6% sucrose/0.15% NaCl LIS¹², but such a distinction could not be made when 10% sucrose LIS was used (Table 1). This finding was unexpected but accords with the known correlation between the immunochemical polymorphism of C4 and functional activity of the molecule¹³. Determination of plasma C4 by single radial immunodiffusion (Behringwerke plates) showed that Ch+, Rg+ plasma usually

contained more C4 than Ch+, Rg- plasma did, and that Ch-, Rg+ plasma contained the least C4. However, when Ch+, Rg- or Ch-, Rg+ plasma could be matched for C4 content with Ch+, Rg+ plasma, the amount of C4, Ch, Rg transferred in 6% sucrose/0.15% NaCl LIS was again correlated with the Ch(Rg) phenotype. This result suggests that there are intrinsic differences in the activity of C4 molecules of different Ch, Rg phenotype.

Previous studies assessing the effects of reducing agents and enzymes¹⁴ on incomplete blood-group antibodies suggested that, in agglutination tests, the Chido antigen on normal red cells is not destroyed by trypsin. We investigated further this apparent localisation of Ch(Rg) to a trypsin-resistant fragment of C4. In a kinetic study, red cells coated with C4, Ch, Rg in 10% sucrose LIS lost all reactivity with anti-C4c within about 2 min of exposure to trypsin at 37 °C, whereas C4d and Ch(Rg) determinants still remained at 60 min (Table 2). C4d and Ch(Rg) determinants were resistant to further cleavage, by chymotrypsin, in conditions in which C4c determinants were lost from cells coated with C4b in LIS (Table 2). The reactions of anti-Ch and anti-Rg with cells treated with trypsin, however, became slightly weaker with time indicating some alteration or destruction of Ch(Rg) with prolonged exposure to the enzyme. This finding correlates with the observation that, in the antiglobulin test, Ch and Rg on normal red cells are much weaker after enzyme-treatment. Since agglutination tests confirmed that Ch(Rg) is borne by a tryptic-C4d fragment of C4, we tested red cells expected to carry C3bINA-cleaved C4d due to complement-activation *in vivo*^{15,16}. Such cells, collected into EDTA from patients with cold autoimmune haemolytic anaemia, failed to react with anti-C4c but were strongly agglutinated by anti-C4d, and by anti-Ch and anti-Rg (Table 2).

In all experiments anti-Ch(Rg) behaved the same as anti-C4d. As the monkey anti-C4d was non-precipitating⁵, it might be directed against few antigenic determinants, possibly just Ch and Rg. In inhibition tests using red cells coated with C4d, Ch, Rg, the anti-C4d was inhibited by Ch+, Rg+ serum but there was no detectable inhibition with Ch-, Rg+ or Ch+, Rg-

serum. Anti-C4d was inhibited by mixing Ch-, Rg+ and Ch+, Rg- serum but the inhibition was less than with Ch+, Rg+ serum. Thus this anti-C4d is predominantly anti-Ch and anti-Rg. This surprising finding presumably reflects the phylogenetic proximity of monkeys to man.

The preservation of Ch and Rg on C4-sensitized red cells exposed to trypsin and chymotrypsin *in vitro*, or C3bINA *in vivo*, indicates that these antigens reside on the limit fragment, C4d, which is stably associated with the cell surface¹⁷. Ch and Rg must, of course, be spatially distant from that portion of C4 which interacts with the membrane. It is interesting that Ch and Rg survive cleavage and presumed conformational change in C4 which precede transfer of the molecule to the red cell. Conformational changes presumably also accompany processing of single-chain precursor C4 (ref. 18) to the mature three-chain structure; anti-Ch(Rg) could be used as a probe for such changes. As the structural locus for C4 (refs. 19, 20) and Ch (ref. 1) and Rg (ref. 2) all map to the same small region of chromosome 6, it is likely that primary structural differences, rather than some obligatory post-translational modification such as glycosylation²¹, generate the Ch, Rg antigenic polymorphism. It does not follow, however, that the electrophoretic polymorphism of C4 is entirely due to the structural differences in C4d which distinguish Ch from Rg. Certainly, in some gel systems^{22,23} the apparent structural variability cannot be explained solely by Ch and Rg differences. The structural polymorphism of C3 is, in fact, in the C3c fragment^{24,25}, and C4 and C3 are homologous proteins.

Graham *et al.*²⁶ have shown that C3d is present on normal red cells. To the extent that normal red cells carry detectable Ch and Rg, these cells are also coated with C4d. A cardinal question, therefore, concerns the origin of the natural Ch(Rg) on red cells. Does natural Ch(Rg) represent low-level sensitization with C4 during physiological activation of the classical complement pathway, or is it bound by a different mechanism? A unified hypothesis that all Ch(Rg) on red cells is dependent on complement activation would accommodate the serological differences between natural Ch(Rg), and Ch(Rg) acquired by

known activation of complement. The nebulous and variable reactions of anti-Ch and anti-Rg with normal red cells are presumably due to only trace amounts of C4d on the cells.

As a number of complement components exhibit polymorphism, there is a potential for formation of alloantibodies to these components by transfusion. However, these components may have to be cell-bound to elicit an immune response. Anti-Ch and anti-Rg, elicited by C4, may be the first examples of alloimmunoglobulins in man.

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Effective subunit vaccines against an enveloped animal virus

VACCINES are available to act against many of the diseases caused by enveloped viruses. The efficacy and safety of the vaccines depend on the method used to convert the virus into a vaccine. Whole viruses that have been attenuated or killed are effective, but both of these types of vaccines may give rise to undesirable side effects which limit their use¹⁻³. These complications are caused by the virus genome or by toxic components in the virus preparations. The components that give rise to the side effects do not seem to be those that are important for evoking protective immunity and it is therefore possible to prepare vaccines which contain only the necessary antigenic subunits, which for the enveloped viruses are the spike glycoproteins⁴⁻⁷. But although subunit vaccines have proved safer

Table 2 Effect of enzymes on C4, Ch(Rg) bound to human red cells

Method of coating red cells with complement	Enzyme treatment of C4-coated red cells	Enzyme	Min	Antiserum		
				Anti-C4c	Anti-C4d	Anti-Ch Rg
LIS	None			>50,000*	>1,600	3,200
<i>in vitro</i>	Trypsin	2	100	>1,600	3,200	3,200
	Trypsin	5	0	>1,600	3,200	3,200
	Trypsin	10	0	>1,600	3,200	3,200
	Trypsin	30	0	>1,600	1,600	1,600
	Trypsin	60†	0	>1,600	1,600	1,600
	Chymotrypsin	30	200	>1,600	3,200	1,600
	Trypsin	30				
	Chymotrypsin	30	0	>1,600	3,200	3,200
Auto-anti-I	None			0	>400	>8
<i>in vivo</i>	Trypsin	30	0	>400	>8	>16
Auto-anti-P	None			0	>400	>8
<i>in vivo</i>	Trypsin	30	0	>400	>8	0

One volume of packed red cells, coated with C4, Ch, Rg in 10% sucrose LIS containing Ch+, Rg+ serum, was incubated at 37°C in four volumes of 1 mg ml⁻¹ Trypsin-TPCK (Worthington Biochemical) in 20 mM Tris-HCl/0.15 M NaCl, pH 7.8 (TBS). At intervals, an aliquot of red cells was removed, centrifuged through a large volume of saline, and washed a further three times. After 30 min, a portion of these cells and cells freshly coated with C4, Ch, Rg were incubated with α -chymotrypsin (Worthington), 1 mg ml⁻¹ TBS containing 10 mM CaCl₂ for 30 min at 37°C, then washed three times. Red cells coated *in vivo* with C4/C3 were obtained from patients with cold autoimmune haemolytic anaemia. In two Ch+, Rg+ patients the auto-antibody had I specificity; in one patient, who was Ch+, Rg-, the auto-antibody had P specificity. The anti-complement reagents used are described in the footnote to Table 1.

* Haemagglutinin titre. † One volume fresh trypsin added at 30 min.

Table 1 Chemical composition of subunit vaccines used

Vaccine preparation	Polypeptide composition	Molecular weight	No. of virus spikes per complex	Lipid content (mg per mg protein)	Detergent content (mg per mg protein)	Infectivity* (PFU)	Ref.
4.5S complexes	E1, E2, E3	1.5×10^5	1	0.01	0.54	NT	16
29S complexes	E1, E2, E3	9.5×10^5	8	0.01	0.03	<10	17
Virosomes	E1, E2, E3	—	~20–2,000†	0.9–1.0	0.05	<10	15

NT, not tested (detergent interferes with assay).

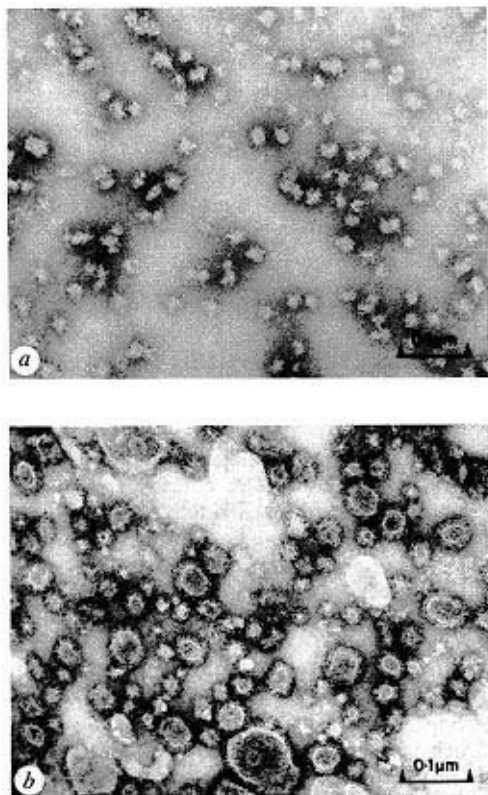
* Infectivity assayed by plaque titration in BHK-21 cells³³ at a protein concentration of 0.2 mg per ml.

† The virosomes consist of a heterogeneous population of vesicles of different sizes.

than whole virus vaccines, their immunogenicity is generally lower^{8,9}, perhaps because the form in which the proteins are presented in the vaccines is not optimal for eliciting a strong immune response¹⁰. In support of that explanation, we present here evidence that a lethal infection of mice with Semliki Forest virus can be prevented by a single vaccination with the virus spike protein, provided it is presented as a micellar aggregate of the protein or as protein reconstituted into vesicles of egg lecithin. The monomer form of the spike protein solubilised with detergent is very much less effective.

Semliki Forest virus (SFV) is well suited for the studies outlined above^{11,12}. It is a simple enveloped virus in the Togaviridae family. In mice lethal strains cause encephalomyelitis which leads to death within a week. The surface glycoproteins of this virus have been studied in detail¹³. Systematic studies of the solubilisation¹⁴ and reconstitution¹⁵ of the virus membrane have yielded methods for the preparation of three different physical forms of the spike protein; (1) the 4.5S complex¹⁶, a complex of one spike protein bound to 75 molecules of Triton X-100; (2) the 29S complex¹⁷, a protein micelle of eight spikes virtually devoid of lipid and detergent and with a molecular weight of 9.4×10^5 ; and (3) virosomes (see ref. 18 for terminology) in which the spike proteins have been reconstituted into vesicles of egg lecithin¹⁵.

Fig. 1 Electron micrographs of *a*, the 29S complexes and *b*, the virosomes used for vaccination. The preparations have been negatively stained.



The vaccine preparations for these studies were made from the prototype strain of SFV which has been passaged in BHK-21 cells several times in our laboratories and is avirulent for mice. Monomers of spike proteins were isolated as 4.5S complexes from the purified virus by disrupting the virus with Triton X-100 and then subjecting this mixture to sucrose gradient centrifugation in the presence of a detergent¹⁶. The proteins obtained are virtually lipid-free and soluble by virtue of the bound detergent. The 29S complexes were prepared by centrifugation of virus disrupted with Triton X-100 into a sucrose gradient, which separates the lipid, detergent and nucleocapsids from the spike proteins¹⁷. These aggregate to octameric complexes which are water soluble (Fig. 1*a*). They are similar in structure to detergent micelles. The protein micelles have a polar surface and the hydrophobic tails of the spike proteins are buried in the interior¹⁹. Other virus spike proteins also aggregate to form soluble complexes (see ref. 19). The virosomes were prepared by the octylglucoside dialysis method¹⁵. Briefly, the virus was solubilised with Triton X-100 and the spike glycoproteins isolated by sedimentation into a sucrose gradient containing the nonionic detergent β -D-octylglucoside. Egg lecithin solubilised with octylglucoside was added (the lipid/protein ratio being 1:1, w/w) and the detergent removed by dialysis (Fig. 1*b*). The properties of the three vaccines are summarised in Table 1.

The amount of 29S complex required to induce protective immunity after a single inoculation into BALB/c mice was studied first. A virus dose of 10^4 plaque forming units (PFU) of the lethal L10 strain¹¹ was used to challenge the mice two weeks after vaccination. The response to increased doses of the vaccine was linear; 0.5 μg of 29S vaccine was sufficient to protect 50% of the mice. A dose of 10 μg induced protection in all animals. Therefore, 10 μg of virus protein was subsequently used in all experiments. We carried out three series of experiments using the three forms of vaccines (Table 2). In the first, the challenge

Table 2 Vaccination against SFV encephalomyelitis in BALB/c mice: the protective effect induced by different forms of purified spike protein vaccine

Physical form of vaccine	Dose (μg protein)	LD ₅₀ of challenge virus (log ₁₀ of PFU)		
		Expt 1	Expt 2	Expt 3
(control)		2.3	2.1	2.6
4.5S complex (monomer)	10	ND	2.7	3.4
29S complex (octamer)	10	>3.5	>5.3	>6.3
Virosome (multimer)	10	>4.0	>5.1	>6.6

The vaccination experiments were performed on 5–6-week-old female BALB/c mice. The mice were vaccinated with a single dose of 10 μg spike protein in 100 μl Eagle's minimal essential medium. One third of the dose was injected subcutaneously and two thirds intraperitoneally (i.p.). Two weeks later groups of 10 mice were challenged i.p. with virulent strain L10.H6.C1 SFV with doses ranging from 50 to 10^4 PFU (Expt 1) 50 to 10^5 PFU (Expt 2) and 50 to 10^7 PFU (Expt 3). The infectivity was determined by plaque titration in BHK-21 cells³³. The amount of protein in the vaccine preparations was measured by the method of Lowry *et al.*³⁴ using 0.1% SDS in the reaction mixture. The LD₅₀ was calculated as described by Spearman and Kärber³⁵. ND, Not determined.

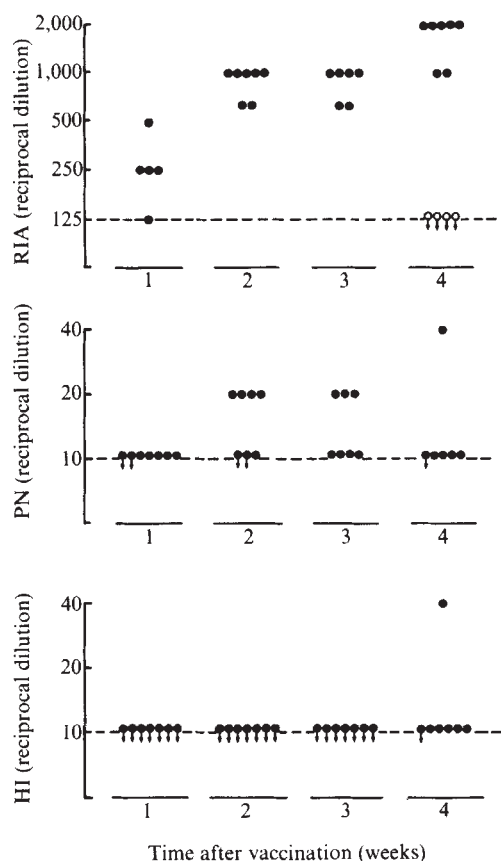


Fig. 2 The antibody response in serum of normal BALB/c and BALB/c nude (*nu/nu*) mice following vaccination with 10 μ g SFV spike protein in virosomes. The sera from normal mice were collected weekly 1–4 weeks after vaccination. The nude mice were challenged with 10^3 PFU virulent virus two weeks after vaccination and after a further two weeks the sera from survivors were collected. The radioimmunoassay (RIA) was carried out in a solid phase system using flexible microtitre plates with U-formed bottom (Cooke no. 22–24). The wells were filled with 1 μ g virus in 80 μ l phosphate-buffered saline (PBS) and incubated overnight at 4 °C. Thereafter the wells were rinsed 3 times with 100 μ l PBS, 30% fetal calf serum and 100 μ l of mouse serum to be assayed was added in different dilutions and incubated for 1 h at room temperature. After washing 100 μ l of immunosorbent purified 125 I anti-mouse γ globulin was added and incubated for 1 h at room temperature. After washing, the wells were cut out and radioactivity determined in a gamma counter (Nuclear Chicago). All sera were tested in triplicate and sera from non-immunised were included as negative controls. The titre was defined as the highest dilution of serum which bound more than 2 times the amount of radioactivity of normal mouse serum controls. The plaque neutralisation (PN) test and the haemagglutination inhibition (HI) test were performed as described previously^{32,36}. ●, Titres from individual normal BALB/c mice; ○, titres from individual nude mice. The arrow indicates that the titre was below the dilution tested. ---, threshold level of the test, that is, the lowest dilution tested.

dose ranged from 50 to 10^4 PFU and in the second and third series the maximum challenge dose was increased to 10^5 and 10^7 PFU, respectively. Even with the highest challenge dose (10^7 PFU) most of the mice vaccinated with the 29S complexes and the virosomes survived. The extent of protection measured by the difference between the LD_{50} in controls and in the groups vaccinated with a single dose of 29S complexes and of virosomes must thus be higher than $10^{3.7}$ (Table 2). We also vaccinated a group of 10 mice twice with virosomes (10 μ g spike protein) two weeks apart and challenged them 14 days later. All the mice survived the highest dose of virus (10^7 PFU).

Mice vaccinated with the 4.5S complex were only slightly protected (Table 2). To verify the very small difference induced

by the 4.5S complex, the non-vaccinated mice and the mice vaccinated with the 4.5S complexes that survived the first challenge dose of virus were given a second challenge of 10^4 PFU of virus (Table 3). All but one of the non-vaccinated mice died, whereas the survival of the vaccinated mice was dependent on the dose of virus given in the first challenge. If the first challenge was higher than one LD_{50} dose ($>10^2$ PFU), the mice survived the second challenge. Thus vaccination with the monomeric form of the spike protein gave a small but detectable protection against the disease.

Table 3 Enhancement by live virus challenge of a poor protective effect induced by vaccination with 4.5S complexes

Vaccine* (day 0)	1° Challenge (day 14)		2° Challenge (day 28)		Comments
	Dose (PFU)	Mortality†	Dose (PFU)	Mortality	
None	$10^{1.5}$	0/10	10^4	10/10	No protection obtained after 1st challenge
	$10^{2.0}$	1/10	10^4	9/9	
	$10^{2.5}$	5/10	10^4	4/5	
	$10^{3.0}$	24/30	10^4	6/6	
4.5 complex (10 μ g protein)	$10^{1.5}$	0/10	10^4	8/10	First challenge with a dose <1 LD_{50}
	$10^{2.0}$	3/10	10^4	5/7	
	$10^{3.0}$	4/10	10^4	0/6	First challenge with a dose >1 LD_{50}
	$10^{4.0}$	8/10	10^4	0/2	

* Vaccination was performed as described in Table 2. First challenge was performed 2 weeks after vaccination, and a second challenge after a further 2 weeks. For details see Table 2.

† Mortality expressed as number of mice killed per total number of group.

The dependence on T cells for induction of protective immunity using virosomes was studied by comparing the response of BALB/c mice with that of BALB/c nude (*nu/nu*) mice (Table 4). The LD_{50} for non-immunised nude mice was higher than for normal BALB/c. In contrast to normal mice no difference in survival time or rate between non-vaccinated and vaccinated mice was observed in *nu/nu* animals. The total serum antibody response was measured with a radioimmunoassay. No antibodies were detected in vaccinated nude mice which were challenged after two weeks and tested after four weeks. In normal mice the serum antibody titres kept rising from the first to the fourth week after immunisation. Antibody against SFV was detectable by plaque neutralisation assays 2 weeks after vaccination, and antibody causing haemagglutination inhibition after four weeks. The titres were low (Fig. 2). The above results suggest that T cells are required for protective immunity, as has been found for several viruses²⁰. More detailed immunological studies are underway to explore the mechanisms of protection against SFV infection.

Previous studies have also shown that solubilisation of togaviruses with different mild detergents leads to vaccines with low efficacy¹². Monomeric forms of spike proteins have been isolated from other enveloped viruses, and they too are poor immunogens. In contrast to SFV spike protein the Friend leukaemia virus spike glycoprotein (gp71) is a typical peripheral membrane protein which does not need detergent for solubility and seems to be attached to the viral membrane through another integral membrane protein²¹. The mice were only partially protected against leukaemia after vaccination with two doses of 100 μ g of spike protein in Freund's adjuvant^{5,21}. A monomer preparation of the influenza virus haemagglutinin has been prepared by releasing the hydrophilic portion of the spike using a protease²². This monomer haemagglutinin was only weakly immunogenic in hamsters and man^{23,24}. These results agree with the known poor immunogenicity of monomeric compared with multimeric forms of proteins^{25–27}. Monomers of spike protein, water soluble with or without detergent, are clearly not suitable as subunit vaccines. One reason why the virosomes and the protein micelles proved so efficient as vaccines is probably due to the multimeric structure of the spike proteins. The virosomes

Table 4 Resistance of vaccinated and non-vaccinated nude and normal BALB/c mice to infection with a lethal strain of SFV

Vaccine	Chal- lenge dose (PFU)	Nude		Normal	
		No. of mice killed per group	Survival time (d)	No. of mice killed per group	Survival time (d)
Virosome	10 ⁴	8/9	9.3 ± 5.9	10 ⁴	0/10
None	10 ⁴	8/10	13.0 ± 5.5	10 ⁴	10/10
Virosome	10 ³	1/5	7	10 ³	0/10
None	10 ³	0/5	—	10 ³	9/10
Virosome	10 ^{2.5}	1/5	6	10 ^{2.5}	0/10
None	10 ^{2.5}	2/5	6	10 ^{2.5}	10/10

Mice were vaccinated with virosomes containing 10 µg spike protein and challenged 14 d later with different doses of virus. For details see Table 2.

contain a high surface density of spike protein similar to the surface of the virus itself. The 29S complexes contain eight spike proteins held together by their hydrophobic ends. Both forms bind effectively to the surface of cells apparently in such a way that spike proteins remain available for interaction with cells of the immune system²⁸. We have recently shown that 29S complexes bind to virus receptors on the cell surface and do not seem to integrate into the cell membrane²⁹. These findings suggest that integration of viral antigens into host cell surface membranes may not be a prerequisite for induction of protective immunity.

Another reason for the effectiveness of these vaccines is probably that mild methods were used to extract the spike proteins from the virus and to prepare the multimeric vaccines. Triton X-100 and octylglucoside are non-ionic detergents known to preserve the biological activities of membrane proteins^{30,31}. Organic solvents and other denaturing agents such as ionic detergents may destroy antigenic determinants on the spike proteins. Whether protein micelles or virosomes will prove efficient as vaccines for other enveloped viruses has yet to be investigated.

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Fibronectin binds to *Staphylococcus aureus*

MANY important activities have been ascribed to fibronectin, which is found in soluble form in many biological fluids and in insoluble form on the surface of fibroblasts^{1–3}. Fibronectin mediates the attachment of fibroblasts and hepatocytes to culture dishes^{4–6} and, when solubilised from the fibroblast surface, it binds to the surface of cultured transformed fibroblasts, restoring their morphology to normal^{7,8}. I now report that the affinity of fibronectin to cell surfaces extends to the prokaryotic cell *Staphylococcus aureus*.

When labelled fibronectin was incubated with both commercial strains and clinical isolates of *S. aureus* up to 70% of the radioactivity bound to the bacteria. *Mycobacterium butyricum*, however, showed only slight binding (Fig. 1). No ¹²⁵I-α-feto protein bound to staphylococci nor did fibronectin bind to formalinised sheep red blood cells.

Table 1 Enrichment of fibronectin with *Staphylococcus aureus*

	Fibronectin concentrations in supernatants and eluates (µg ml ⁻¹)			
	Expt 1		Expt 2	
	Supernatant	Eluate	Supernatant	Eluate
<i>S. aureus</i>	0.1	11.5	0.3	12.5
<i>M. butyricum</i>	6.2	0.6	4.6	0.8
Control*	ND	ND	2.9	0.7

Citrated human plasma, 10–20 µl, containing 200–500 µg ml⁻¹ fibronectin was diluted to 0.5 ml with phosphate buffered saline (PBS) and incubated with 0.5 ml 10% suspension of bacteria overnight. Supernatants were collected after centrifugation at 1,000 g for 30 min, and the bacteria were washed two times with 2.0 ml PBS. Fibronectin bound to bacteria was eluted adding 150 µl 8 M urea containing 0.05 M Tris, pH 8.0, and fibronectin was quantitated by radioimmunoassay. ND, not determined.

* Control tubes were processed as above without any bacteria.

Staphylococcal binding was confirmed using unlabelled fibronectin. After incubation of normal human plasma with *S. aureus* and *M. butyricum*, radioimmunoassay⁹ showed marked enrichment of fibronectin in eluates of staphylococci but not in eluates of mycobacteria or in control eluates (Table 1).

To characterise the binding, various substances were tested for the capacity to inhibit binding of ¹²⁵I-fibronectin to staphylococci. As expected, unlabelled fibronectin was inhibitory, giving half maximal inhibition at 3–4 µg per ml of purified fibronectin. Inhibition by plasma samples containing known amounts of fibronectin was compatible with inhibition by purified fibronectin in the absence of plasma components (Fig. 2). Of the sugars tested, only nonacetylated amino sugars were inhibitory. Half maximal inhibition was obtained with 0.5 M solutions (Table 2). L-Lysine and urea were also inhibitory, whereas divalent cations or chelating agents had no effect on binding. Denatured collagen showed a maximal binding of only 18%, which was not concentration-dependent between 100 µg ml⁻¹ and 5 mg ml⁻¹. This slight inhibition might be due to steric inhibition by collagen molecules bound to fibronectin. Protein A, which is present in the cell wall of *Staphylococcus aureus* and binds to immunoglobulins, did not inhibit at 100 µg ml⁻¹. Also F(ab')₂ fragments of purified anti-fibronectin antibodies failed to inhibit staphylococcal binding but inhibited the binding of fibronectin to collagen-Sepharose (data not shown). F(ab')₂ fragments were used rather than whole antibodies to avoid the binding between protein A and Fc fragments of antibodies.

Thus the binding of fibronectin to *S. aureus* resembles the binding of cells to collagen-coated culture dishes, a process in which fibronectin has been shown to mediate attachment^{4,5,10}. Like staphylococcal binding, attachment to culture dishes is

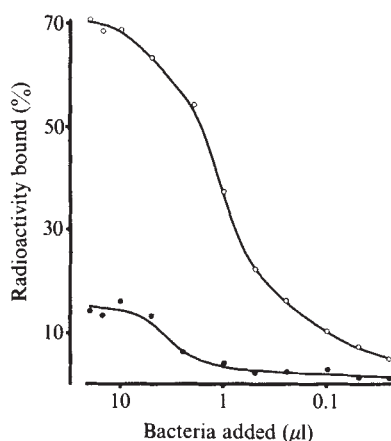


Fig. 1 Binding of ¹²⁵I-human fibronectin to fixed *S. aureus* (O) and *M. butyricum* (●). Fibronectin was isolated from normal citrated human plasma using affinity chromatography on denatured collagens¹¹ and radioiodinated using the chloramine-T method⁹. The labelled fibronectin showed a sharp peak of radioactivity co-migrating with the unlabelled fibronectin (MW 220,000) in SDS-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol¹². Commercially available heat-killed and formalin-fixed *S. aureus* (Pansorbin, Calbiochem) and *M. butyricum* (Difco) were used. *S. aureus* isolated from routine clinical specimens, heat-killed and fixed with 10% trichloroacetic acid was also used in some experiments, with the same results. The bacteria were washed once with 5 volumes of 4.0 M urea solution containing 0.05 M Tris, pH 7.5, twice with 15–20 times the volume of buffer without urea, and used as a 10% suspension (v/v) in PBS containing 0.1% bovine serum albumin (BSA). Decreasing amounts of 10% bacterial suspension were adjusted to 1.0 ml with PBS-BSA, and ¹²⁵I-human fibronectin (1.0–1.5 × 10⁴ c.p.m.) was added in 50 µl. After incubation overnight at room temperature under continuous rotation, the bacteria were isolated by centrifugation (1,000g for 30 min), washed once with 1.0 ml PBS-BSA, and transferred in 250 µl of PBS-BSA into new tubes for counting.

Each point represents the mean of duplicate tubes.

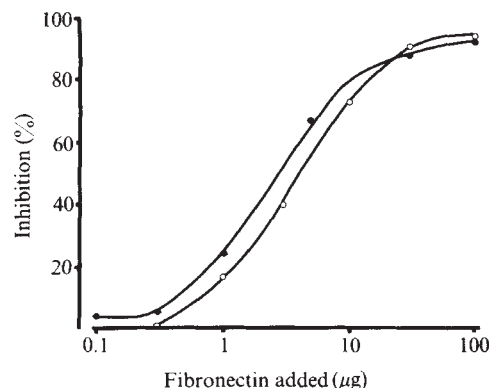


Fig. 2 Inhibition of binding of ¹²⁵I-human fibronectin to *S. aureus* with normal human plasma (●) and fibronectin isolated from human plasma (O). Increasing amounts of normal human plasma, containing 210 µg ml⁻¹ fibronectin, and isolated human plasma fibronectin were incubated with 50 µl of a 10% bacterial suspension. This amount of suspension gave about 50% of maximal binding. The test was carried out as for Fig. 1. Inhibition percentages were calculated according to the formula:

$$\frac{[\text{Binding \% without inhibitor} - \text{binding \% with inhibitor}]}{\text{Binding \% without inhibitor}} \times 100.$$

inhibited by amino sugars and lysine, although divalent cations are not required for the process. The lack of inhibition of staphylococcal binding in the presence of denatured collagen shows that this binding is not related to the binding of fibronectin to collagen¹¹.

This conclusion is further supported by the observation that F(ab')₂ fragments of anti-fibronectin antibodies do not inhibit staphylococcal binding but inhibit and reverse the binding of fibronectin to collagen⁹. Whether fibronectin also binds to living *S. aureus* and whether it is the anti-staphylococcal factor remains to be seen. If staphylococcal binding is an expression of the affinity of fibronectin for cell surfaces, it could prove useful in the elucidation of the nature of the cellular receptor(s) involved.

Table 2 Inhibition of binding of labelled human fibronectin to *Staphylococcus aureus*

Inhibitor	Concentration needed to inhibit binding (mM)	
	20%	50%
D-Glucosamine ^a	150	500
D-Galactosamine ^a	ND	500
L-Lysine ^b	100	>1,000
NaCl	100	>2,000
Urea	600	1,050
Anti-human fibronectin F(ab') ₂ (µg ml ⁻¹)	>190	—

Binding assay and calculations for inhibition percentages were as described in Figs 1 and 2. The solutions were neutralised with NaOH (a) or with HCl (b) before incubation with bacteria. Amino sugars were used in HCl form, and they were neutralised to pH 7.5 with NaOH before testing. Conductivity measurements showed that 0.5 M stock solutions of these sugars contained the equivalent of 0.2 M NaCl above the conductivity of PBS-bovine serum albumin-buffer alone. This amount of NaCl inhibited the binding 28%. F(ab')₂ fragments of purified rabbit anti-human fibronectin antibodies were obtained as follows: 2.4 mg purified antibodies were incubated with 500 µg pepsin in 0.1 M sodium acetate buffer, pH 4.5, overnight at room temperature. Digestion was stopped by dialysis against 0.05 M Tris-HCl buffer, pH 8.0, and the digest was passed through a Sepharose-protein A column. Protein peak consisting of unbound material was collected and used in the tests. No inhibition was obtained with the following substances: 1 mM MgCl₂ and CaCl₂, 100 mM EDTA and EGTA, 0.5 M D-glucose, D-galactose, D-N-acetylglucosamine, D-N-acetylgalactosamine, L- and D-fucose, D-mannose, L-rhamnose, L- and D-xylose, 0.25 M D-N-acetylneuraminic acid and 1 M glycine. ND, not determined.

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The amino acid sequence of *Physarum* actin

THE realisation that actin is involved not only in contractility in muscle tissue but also in general cytoplasmic movement and cell locomotion was based on the isolation of actin from lower eukaryotic organisms, such as *Physarum polycephalum*¹, which show active protoplasmic streaming. It is now accepted that actin is the major structural protein of microfilaments characteristically found in the cytoplasm of most if not all higher cells, and that actins have been highly conserved during eukaryotic evolution and differentiation due to the constraints imposed by the large number of specific interactions which actin shows with other cytoplasmic proteins (for a review see ref. 2). Preliminary fingerprint analysis on *Physarum* actin³ and partial amino acid sequence analysis on actin from *Acanthamoeba* (ref. 4 and Elzinga cited in ref. 2) have emphasised this point, although so far a complete amino acid sequence has not been reported for actin from a lower eukaryotic species. Our studies on the primary structure of different mammalian actins have shown that several cytoplasmic actins are very similar if not identical in amino acid sequence, although they differ by at least 25 amino acids from the corresponding skeletal muscle actin⁵. The availability of fast amino acid sequence procedures for actin⁵ led us to study the actin from *Physarum polycephalum* to assess how cytoplasmic actins differ between lower and higher eukaryotes. We report here results which show that *Physarum* plasmodia, in contrast to non-muscle cells of higher vertebrates⁵⁻⁷, contain only one cytoplasmic actin species. This actin is more closely related to mammalian cytoplasmic actins than these actins are to skeletal muscle actin. The high degree of structural conservation of the actin sequence and structure during eukaryotic evolution is illustrated by the absence of any amino acid deletions or additions to the polypeptide chain beyond residue 2. Furthermore, the distribution of the proline residues remains unchanged and substitutions of amino acid residues with charged side chains are very rare, indicating a high conservation of the surface topography of the actin molecule.

Actin was purified from an acetone powder prepared from *Physarum* plasmodia. A low salt extract was chromatographed on deoxyribonuclease I immobilised on Sepharose 4B as described elsewhere^{5,8}. The resulting actin preparation was at least 95% pure as judged by polyacrylamide gel electrophoresis in the presence of dodecyl sulphate, where *Physarum* actin coelectrophoreses with mammalian actins. In addition, isolectric focusing analysis in the presence of urea and NP40

revealed a single protein species, focusing, in agreement with previous studies⁹, very close to although at a slightly more acidic pH than rabbit skeletal muscle actin. *Physarum* actin was processed through performic acid oxidation and tryptic digestion as described for other actins^{5,10}. Peptides were separated by the preparative two-dimensional 'fingerprint' system on Whatman 3MM paper which we have described in detail previously^{5,10,11}. The insoluble tryptic 'core' was digested with thermolysin and the resulting peptides purified as described elsewhere⁵. This system accounts by tryptic and thermolytic peptides for the full amino acid sequence of the actin polypeptide chain⁵. All peptides were characterised by their electrophoretic and chromatographic mobilities, their amino acid composition and their behaviour in the Offord plot¹². Peptides of > 10 amino acid residues were further fragmented by secondary enzymatic digestion and the isolated fragments characterised as above. Amino acid substitutions indicated in the resulting proteolytic fragments were localised by manual dansyl-Edman degradation and/or by degradation with leucine aminopeptidase and carboxypeptidase Y. A full description of the actin sequence methodology is presented elsewhere⁵.

Figure 1 shows the complete amino acid sequence of *Physarum* actin. The order of the tryptic and thermolytic peptides in the actin polypeptide chain is based on homology with the rabbit skeletal muscle actin sequence¹³. The polypeptide chain contains 375 amino acid residues although the numbering system indicates only 374. This is because a serine residue following residue 234 has been found in all cytoplasmic actins so far studied^{5,14} and has probably been overlooked in the original rabbit skeletal muscle actin sequence^{13,14}. To avoid renumbering of the previously documented amino acid exchange past this residue, we agree with the proposal¹⁴ to indicate this residue as serine 234a.

Table 1 summarises those amino acid exchanges which differentiate *Physarum* actin (Fig. 1) from mammalian cyto-

Fig. 1 Amino acid sequence of actin from plasmodium of *Physarum polycephalum*. X indicates a blocking group which is probably the acetyl group found in other actins¹³. His^{7Me} is N⁷-methylhistidine. For methods on sequence determination, see ref. 5 and text. Residues which are underlined differ from the corresponding amino acid residue in mammalian γ -cytoplasmic actin⁵. Alignment of the *Physarum* actin peptides was based upon comparison with the rabbit skeletal muscle actin sequence¹³.

X-Glu-Gly-Glu-Asp-Val-Gln-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Met-Cys-Lys-Ala-Gly-	10	20
Phe-Ala-Gly-Asp-Asp-Ala-Pro-Arg-Ala-Val-Phe-Pro-Ser-Ile-Val-Gly-Arg-Pro-Arg-His-	30	40
Thr-Gly-Val-Met-Val-Gly-Met-Gly-Gln-Lys-Asp-Ser-Tyr-Val-Gly-Asp-Glu-Ala-Gln-Ser-	50	60
Lys-Arg-Gly-Ile-Leu-Thr-Leu-Lys-Tyr-Pro-Ile-Glu-His-Gly-Ile-Val-Thr-Asn-Trp-Asp-	70	80
Asp-Met-Glu-Lys-Ile-Trp-His-His-Thr-Phe-Tyr-Asn-Glu-Leu-Arg-Val-Ala-Pro-Glu-Glu-	90	100
His-Pro-Val-Leu-Leu-Thr-Glu-Ala-Pro-Leu-Asn-Pro-Lys-Ala-Asn-Arg-Glu-Lys-Met-Thr-	110	120
Gln-Ile-Met-Phe-Glu-Thr-Phe-Asn-Thr-Pro-Ala-Met-Tyr-Val-Ala-Ile-Gln-Ala-Val-Leu-	130	140
Ser-Leu-Tyr-Ala-Ser-Gly-Arg-Thr-Thr-Gly-Ile-Val-Met-Asp-Ser-Gly-Asp-Gly-Val-Ser-	150	160
His-Thr-Val-Pro-Ile-Tyr-Glu-Gly-Tyr-Ala-Leu-Pro-His-Ala-Ile-Leu-Arg-Leu-Asp-Leu-	170	180
Ala-Gly-Arg-Asp-Leu-Thr-Asp-Tyr-Leu-Met-Lys-Ile-Leu-Thr-Glu-Arg-Gly-Tyr-Ser-Phe-	190	200
Thr-Thr-Thr-Ala-Glu-Arg-Glu-Ile-Val-Arg-Asp-Ile-Lys-Glu-Lys-Leu-Ala-Tyr-Val-Ala-	210	220
Leu-Asp-Phe-Glu-Gln-Glu-Met-Gln-Thr-Ala-Ala-Ser-Ser-Ser-Ala-Leu-Glu-Lys-Ser-Tyr-	230	234a
Glu-Leu-Pro-Asp-Gly-Gln-Val-Ile-Thr-Ile-Gly-Asn-Glu-Arg-Phe-Arg-Cys-Pro-Glu-Ala-	250	260
Leu-Phe-Gln-Pro-Ser-Phe-Leu-Gly-Met-Glu-Ser-Ala-Gly-Ile-His-Glu-Thr-Thr-Tyr-Asn-	270	280
Ser-Ile-Met-Lys-Cys-Asp-Val-Asp-Ile-Arg-Lys-Asp-Leu-Tyr-Gly-Asn-Val-Val-Leu-Ser-	290	300
Gly-Gly-Thr-Thr-Met-Phe-Pro-Gly-Ile-Ala-Asp-Arg-Met-Gln-Lys-Glu-Leu-Thr-Ala-Leu-	310	320
Ala-Pro-Ser-Thr-Met-Lys-Ile-Lys-Ile-Ile-Ala-Pro-Pro-Glu-Arg-Lys-Tyr-Ser-Val-Trp-	330	340
Ile-Gly-Gly-Ser-Ile-Leu-Ala-Ser-Leu-Ser-Thr-Phe-Gln-Gln-Met-Trp-Ile-Ser-Lys-Glu-	350	360
Glu-Tyr-Asp-Glu-Ser-Gly-Pro-Ser-Ile-Val-His-Arg-Lys-Cys-Phe-	370	

Table 1 Positions in the amino acid sequence at which exchanges have been detected between actins from *Physarum polycephalum*, mammalian non-muscle tissue and rabbit skeletal muscle

Position	Mammalian cytoplasmic actin		<i>Physarum polycephalum</i> actin*	Rabbit skeletal muscle actin†
	β	γ		
1	Absent		<u>Glu</u>	<u>Asp</u>
2	Asp	Glu	<u>Gly</u>	<u>Glu</u>
3	Asp	Glu	<u>Glu</u>	<u>Asp</u>
4	Asp	Glu	<u>Asp</u>	<u>Glu</u>
5	Ile		<u>Val</u>	<u>Thr</u>
6	Ala		<u>Gln</u>	<u>Thr</u>
10	Val	Ile	<u>Ile</u>	<u>Cys</u>
16	Met		<u>Met</u>	<u>Leu</u>
17	Cys		<u>Cys</u>	<u>Val</u>
41	Gln		<u>Thr</u>	<u>Gln</u>
76	Val		<u>Val</u>	<u>Ile</u>
103	Val		<u>Val</u>	<u>Thr</u>
129	Thr		<u>Thr</u>	<u>Val</u>
153	Met		<u>Met</u>	<u>Leu</u>
160	Thr		<u>Ser</u>	<u>Thr</u>
162	Thr		<u>Thr</u>	<u>Asn</u>
176	Leu		<u>Leu</u>	<u>Met</u>
201	Thr		<u>Thr</u>	<u>Val</u>
217	Cys		<u>Ala</u>	<u>Cys</u>
225	Gln		<u>Gln</u>	<u>Asn</u>
228	Ala		<u>Gln</u>	<u>Ala</u>
234a	Ser		<u>Ala</u>	<u>Ser</u>
259	Ala		<u>Ala</u>	<u>Thr</u>
266	Leu		<u>Leu</u>	<u>Ile</u>
271	Cys		<u>Ala</u>	<u>Ala</u>
278	Phe		<u>Tyr</u>	<u>Tyr</u>
286	Val		<u>Val</u>	<u>Ile</u>
294	Ala		<u>Gly</u>	<u>Ala</u>
296	Thr		<u>Val</u>	<u>Asn</u>
298	Leu		<u>Leu</u>	<u>Met</u>
305	Tyr		<u>Phe</u>	<u>Tyr</u>
316	Ile		<u>Leu</u>	<u>Ile</u>
357	Ser		<u>Ser</u>	<u>Thr</u>
359	Gln		<u>Glu</u>	<u>Gln</u>
364	Ser		<u>Ser</u>	<u>Ala</u>

Data on the amino acid sequences of the mammalian cytoplasmic actins are taken from previous studies^{5,10}, those for rabbit skeletal muscle actin are derived from ref. 13 with minor corrections^{5,14}. Amino acid residues given in separate columns for the mammalian cytoplasmic actins are found in the β and γ forms, respectively^{5,10}. The numbering system using 234a is taken from ref. 14 (see also ref. 5 and text).

* Residues underlined indicate the 17 positions where *Physarum* actin differs from mammalian γ cytoplasmic actin.

† Residues underlined indicate the 25 positions where mammalian skeletal muscle actin differs from mammalian cytoplasmic actin.

plasmic actins⁵ and from rabbit skeletal muscle actin¹³, with the latter sequence containing the few minor corrections recently proposed^{5,14}. Like all other actins, *Physarum* actin contains 3-methylhistidine at position 73. We have not been able to detect *N*-methyllysine residues in *Physarum* actin, although they have been reported to occur in *Acanthamoeba* actin¹⁵.

The results presented in Table 1 indicate the strict conservation of the actin structure during evolution. *Physarum* actin and mammalian γ -cytoplasmic actin differ in only 4% of their primary structure (see the 17 positions indicated in Table 1). Comparison with rabbit skeletal muscle actin reveals 8% differences. We previously reported a 7% difference in primary structure in the comparison of mammalian cytoplasmic actins and skeletal muscle actin⁵ (see the 25 positions indicated in Table 1). Beyond the six N-terminal residues which are highly variable in all actins so far studied^{10,16}, *Physarum* actin differs from mammalian cytoplasmic actins in only 12 residues out of a total of 369 residues (a difference of approximately only 3.2%). As was the case for different mammalian actins^{5,10,16}, the few amino acid exchanges involving charged residues are concentrated in the far N-terminal region of the actin polypeptide chain. In addition, however, *Physarum* actin shows a single

Gln→Glu substitution (residue 359) past the first four N-terminal residues. The partial sequence data⁴ on *Acanthamoeba* actin indicate another rare substitution involving a charged residue, that is, residue 228 shows an Ala→His exchange. As *Physarum* actin is characterised by a Gln in this position, it seems possible that this residue is rather variable. In the remainder of the currently available sequence data *Acanthamoeba* actin (ref. 4 and Elzinga cited in ref. 2) and *Physarum* actin are rather similar and so far only five amino acid substitutions (residues 76, 103, 106, 228 and 359) have been found.

Physarum actin differs by isoelectric focusing analysis from mammalian cytoplasmic β and γ actins and behaves very similarly to skeletal muscle actin (α -actin)⁹. The sequence data support this result in general, as Glu 359 gives for *Physarum* actin and skeletal muscle actin the same total number of acidic and basic amino acid residues, whereas the more basic mammalian cytoplasmic actins show one less acidic residue (for a discussion of isoelectric points of different actins see refs 5–7, 10). However, we stress that skeletal muscle actin has one more aspartic acid residue and one less glutamic acid residue than *Physarum* actin. Therefore, one could expect a slightly higher isoelectric point for *Physarum* actin, whereas a slightly lower isoelectric point is found experimentally. We cannot explain this small difference, although it could be due to some subtle influence by amino acid changes next to some charged residues, or to the interruption of the four continuous acidic residues at the N-terminus of muscle actin on transition to *Physarum* actin.

The amino acid sequences of several actins from higher vertebrates^{5,10} together with the sequence of *Physarum* actin explain why several antiactin antibodies show no species specificity and cover species as divergent as man and *Dictyostelium*¹⁷ or *Physarum* (K.W., unpublished observation).

Are the 17 observed amino acid exchanges between *Physarum* actin and mammalian cytoplasmic actin functionally important or are they solely the result of evolutionary drift? Studies by Korn *et al.*^{2,18} show that the vertebrate cytoplasmic F-actins differed from the *Acanthamoeba* F-actin in their ability to activate the Mg²⁺ ATPase of rabbit skeletal muscle heavy meromyosin. Thus, our working hypothesis is that some of the amino acid exchanges found between *Physarum* and mammalian cytoplasmic actins will have an important role in the interaction between actin and myosin. Future experiments are aimed at the identification of these residues.

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Bromodeoxyuridine mutagenesis in mammalian cells is stimulated by thymidine and suppressed by deoxycytidine

THE most widely accepted explanation for the mutagenicity of 5-bromodeoxyuridine (BUdR) is that mutations occur as the result of occasional mispairing of the analogue during DNA replication^{1,2}, although the correlation between mutagenesis and the amount of bromouracil (BU) incorporated into DNA has been questioned^{3,4}. We have previously shown that BUdR mutagenesis in Syrian hamster cells (measured by the induction of ouabain-resistant (OB^r) or 6-thioguanine-resistant (TG^r) mutants) is determined by the concentration of BUdR in the medium rather than the amount of BU in DNA⁵. This suggested that BUdR nucleotides are involved in BUdR mutagenicity in mammalian cells⁵ (see also ref. 4). BUdR triphosphate (BUdRTP) inhibits the ribonucleotide reductase-catalysed reduction of cytidine diphosphate to deoxycytidine (CdR) diphosphate⁶, as does thymidine (TdR) triphosphate (dTTP)^{7,8}. Therefore, we have investigated the effects of CdR and TdR on BUdR mutagenesis, and report here that the former suppresses and the latter stimulates this process.

The cell lines used have been previously described. RPMI 3460 is a pigmented Syrian hamster melanoma line⁹, and HAB-2E is a line derived from 3460 by selection for viability with all the thymine residues in nuclear DNA replaced by BU^{5,10}. Most of the experiments were carried out with HAB-2E cells and OB^r as the marker for mutagenesis. Table 1 (expt 1) shows that the addition of 1 μ M CdR decreased BUdR mutagenicity and that the maximum decrease was obtained with 10 μ M CdR. The addition of 10 μ M CdR also decreased BU incorporation into DNA (BU substitution), presumably due to the intracellular conversion of the CdR to TdR nucleotides¹². However, the maximum suppression of mutagenicity was observed when the CdR concentration was increased from 3 to 10 μ M, over which range there was no change in BU substitution. Similar results were obtained (Table 1, expt 2) in the presence of aminopterin,

Fig. 1 Effect of BUdR concentration on the suppression of mutagenicity by CdR. HAB-2E cells were grown in E medium containing 0.1 mM hypoxanthine and 0.4 μ M aminopterin, plus BUdR and TdR in a 4:1 ratio. The cells were grown either in the presence (■) or absence (●) of 200 μ M CdR. The frequency of OB^r mutants was determined and expressed as described in Table 1. The numbers next to the data points indicate the levels of BU substitution for those points, as described in Table 1.

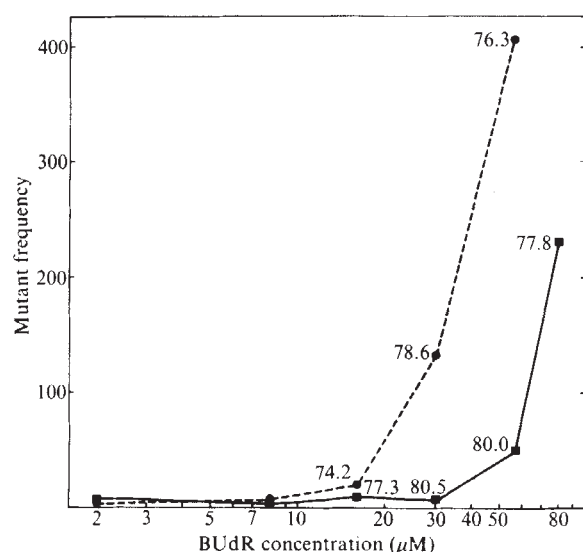


Table 1 Suppression of BUdR mutagenesis by CdR

Aminopterin	Additive (μ M)			BU substitution (%)	Mutant frequency
	BUdR	TdR	CdR		
Expt 1					
—	—	30	—	—	<0.5
—	30	—	—	72.3	349
—	50	12.5	—	67.7	1,194
—	50	12.5	1	63.2	397
—	50	12.5	3	58.0	221
—	50	12.5	10	58.1	37
—	50	12.5	30	57.7	30
—	50	12.5	100	55.4	46
Expt 2					
—	—	30	—	—	<0.3
—	30	—	—	71.3	295
+	50	12.5	—	77.5	463
+	50	12.5	1	ND	252
+	50	12.5	3	ND	262
+	50	12.5	10	78.6	82
+	50	12.5	30	ND	83
+	50	12.5	100	ND	105

HAB-2E cells were maintained routinely in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (E medium). For mutagenicity assays, Falcon plastic tissue culture flasks (75 cm²) were inoculated with 5×10^5 cells in 10 ml of E medium containing the indicated additives. When aminopterin was used, it was present at 0.4 μ M and 0.1 mM hypoxanthine was also added. At 3 and 6 d after inoculation, the medium was renewed with 50 ml fresh medium containing the same additives. After 1 week in BUdR-containing medium, the cells were collected and flasks (75 cm²) were reinoculated with 5×10^5 cells in 10 ml of E medium containing 0.1 mM hypoxanthine and 16 μ M TdR (HT medium). They were cultured in HT medium for 1 week to allow the expression of induced mutations, and then collected and tested for drug resistance. Cells were protected at all times from exposure to light with wavelengths below 550 nm (ref. 11), because of the increased photosensitivity of cells with BU-substituted DNA. During the exposure of cells to BUdR for mutagenesis, the level of BU incorporated into nuclear DNA was determined as previously described⁵. The amount of BU in DNA is expressed as 'BU substitution', defined as the % of thymine residues in nuclear DNA replaced by BU. The results are the mean of four determinations per sample; the s.e.m. was generally $\leq \pm 0.5$. The frequency of OB^r mutants was determined by inoculating 3–5 tissue culture dishes (150 mm diameter) each with 10^6 cells in E medium containing 0.9 mM OB. Plating efficiencies were determined by inoculating 3 dishes (60 mm diameter) each with 250 cells in E medium alone. Seven or eight days after inoculation, the dishes were fixed with methanol and stained with Giemsa for colony counting. The mutant frequencies are expressed as the number of OB^r colonies per 10^6 cells and are corrected for the plating efficiency of the cells in the absence of OB. ND, not determined.

which completely blocks the conversion of CdR to TdR nucleotides¹². Thus, CdR suppresses BUdR mutagenicity without being converted to TdR nucleotides and without altering the amount of BU in DNA.

The effect of increasing the BUdR concentration in the presence of a fixed concentration of CdR was also determined (Fig. 1). HAB-2E cells were exposed to BUdR and TdR in a 4:1 ratio in the presence of aminopterin. In the absence of CdR, BUdR mutagenicity occurred when the BUdR concentration exceeded 10 μ M. However, in the presence of CdR, BUdR mutagenicity did not occur until the BUdR concentration exceeded 30 μ M. The increase in mutant frequency with increasing BUdR seemed to occur at approximately the same rate in the presence of CdR as in its absence, although at higher BUdR concentrations. Apparently, a 2.5-fold increase in BUdR concentration is sufficient to overcome the suppression of mutagenicity by CdR completely. Note that none of these effects was associated with changes in BU substitution.

As dTTP, like BUdRTP, can perturb CdR metabolism, the effects of TdR on BUdR mutagenicity were tested. Table 2 (expt 1) shows that the addition of 10 μ M TdR to 10 μ M BUdR (a

relatively nonmutagenic concentration) increased the frequency of mutants while decreasing BU substitution. The addition of 100 μ M TdR resulted in a similar stimulation of mutagenesis, even though the BU substitution dropped to a very low level. In addition, the stimulation by TdR was suppressed by CdR. Table 2 (expt 2) shows that TdR was also stimulatory to mutagenesis in the presence of aminopterin. CdR again suppressed not only BUdR mutagenicity but also TdR stimulation, and the CdR effects were not associated with changes in BU substitution. Because TdR stimulated mutagenesis, the mutagenicity of TdR alone was tested, but, up to 700 μ M, was nonmutagenic (Table 2, expt 1). (The demonstration that TdR stimulated BUdR mutagenesis in these cells is at variance with studies on prokaryotes, in which thymine suppresses BU mutagenesis.)

Table 2 Stimulation of BUdR mutagenesis by TdR

Aminopterin	Additive (μM)			BU substitution (%)	Mutant frequency
	BUdR	TdR	CdR		
Expt 1					
—	—	30	—	—	<0.4
—	30	—	—	68.1	119
—	30	—	200	36.9	22
—	10	—	—	31.0	4.5
—	10	—	200	18.3	<0.3
—	10	10	—	26.2	19
—	10	10	200	15.3	0.7
—	10	30	—	16.7	13
—	10	30	200	11.9	1.7
—	10	100	—	7.4	22
—	10	100	200	5.6	0.7
—	—	700	—	—	<0.3
Expt 2					
—	30	—	—	65.3	281
+	10	—	—	96.7	31
+	10	—	200	96.7	6.0
+	10	10	—	40.8	39
+	10	10	200	38.7	0.4
+	10	30	—	19.5	94
+	10	30	200	20.5	3.9
+	10	100	—	8.4	93
+	10	100	200	7.2	1.4

HAB-2E cells were assayed for mutagenicity as described in Table 1, as are methods for determination of BU substitution and mutant frequency.

Experiments were carried out, using resistance to 18 μ M TG as the marker, to determine whether the above results were applicable to mutations other than OB^r. Experiments were also carried out using the parental melanoma line 3460, to determine whether the above conclusions were applicable to wild-type cells as well as HAB-2E. The results of these experiments agreed completely with those described above (data not shown).

The results presented above suggest that interactions involving small molecules play a central part in BUdR mutagenesis in mammalian cells. We have shown that (1) CdR suppresses BUdR mutagenicity, (2) CdR suppression can occur without the conversion of CdR to TdR nucleotides and without any change in the amount of BU in DNA, (3) TdR stimulates the mutagenicity of BUdR, while decreasing the amount of BU in DNA, and (4) the stimulation by TdR is suppressed by CdR. These results were observed for two markers of mutagenesis and for two cell lines.

The molecular mechanisms underlying the effects of CdR and TdR on BUdR mutagenesis remain to be elucidated. However, both BUdRTP and dTTP inhibit the ribonucleotide reductase-catalysed reaction which generates CdR nucleotides. Thus, BUdR mutagenicity in mammalian cells, and its enhancement by TdR, may involve the perturbation of CdR metabolism. As mutagenesis can occur even in the presence of very high concentrations of CdR, the results cannot be explained by

simple CdR starvation. In addition, as TdR by itself is not mutagenic, it seems that simply perturbing CdR metabolism in the absence of BUdR does not cause mutations. (Our results do not agree with a recent report of mutagenicity by TdR alone¹³.) Apparently, some BUdR is necessary to TdR stimulation of mutagenesis to occur, but only a relatively small amount of BUdR is sufficient.

One possible mechanism for the role of CdR metabolism in BUdR mutagenesis is that mutagenesis could involve an 'error-prone' repair system¹⁴ induced by the perturbation of CdR metabolism. It is also possible that altered CdR metabolism could cause an increased error frequency during DNA replication, due to allosteric changes in DNA polymerase itself (compare ref. 4) or to an imbalance of the deoxyribonucleoside triphosphates^{13,15}. Although our results demonstrate that BUdR mutagenicity involves effects at sites outside DNA, they do not indicate whether there must also be some BU present in DNA to act as a substrate for any mutagenic functions induced.

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Stimulation of DNA synthesis by tumour promoter and pure mitogenic factors

12-O-TETRADECANOYL PHORBOL-13-ACETATE (TPA), a potent tumour-promoting agent, stimulates the proliferation of various cell types, including fibroblasts, in cell culture (see ref. 1 for review). Recent studies have demonstrated that there is a close correlation between the potency of phorbol esters for promoting skin tumours and their ability to stimulate DNA synthesis in cultured cells². The growth-stimulating activity of TPA is completely dependent on the presence of serum in the nutrient medium. This requirement for serum has been demonstrated in cultures of mouse epidermal cells³ and fibroblastic cells^{4,5}. The role of serum in the cellular response to TPA remains unresolved. We report here that serum can be replaced by polypeptide hormones like epidermal growth factor (EGF)⁶, and insulin and by the growth factor isolated from the conditioned medium of SV40 BHK cells (fibroblast-derived growth factor, FDGF)⁷. Low concentrations of TPA and the growth-

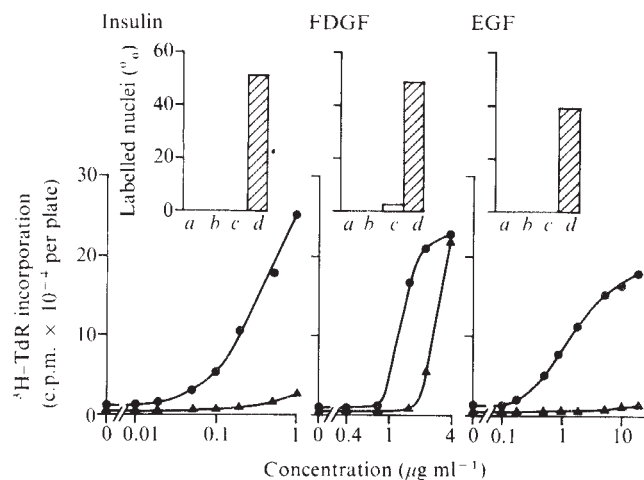
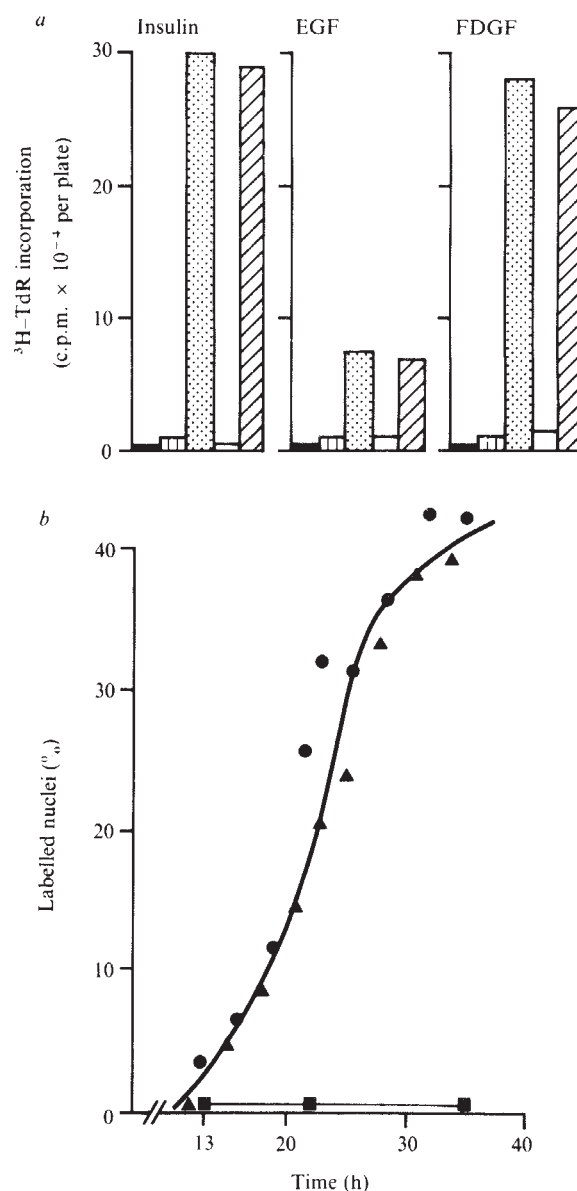


Fig. 1 Effect of insulin, FDGF or EGF on DNA synthesis in the absence (▲) or in the presence of $0.1 \mu\text{g ml}^{-1}$ TPA (●). Cultures were exposed to the peptide with or without TPA in 2 ml of a 1:1 mixture of Dulbecco's modified Eagles' medium (DMEM) and Waymouth's¹⁴ medium containing $^3\text{H-TdR}$ ($0.5 \mu\text{Ci ml}^{-1}$; $0.5 \mu\text{M}$). After 40 h at 37°C , the radioactivity incorporated into acid-precipitable material was measured as previously described¹⁵ (10% foetal calf serum produced $^3\text{H-TdR}$ incorporation of 26.5×10^4 , 27.5×10^4 and 30.7×10^4 c.p.m. respectively, for the experiments involving insulin, FDGF and EGF). All points are the average of duplicate plates. Stock 3T3 cells were passaged at 3-d intervals in DMEM containing 10% foetal calf serum— 100 U ml^{-1} penicillin and 100 ng ml^{-1} streptomycin in a humidified 10% CO_2 atmosphere at 37°C . In all experiments, cell suspensions were plated in 30-mm Nunc dishes in DMEM supplemented with 10% foetal calf serum, re-fed with the same medium after 3 d, and kept for at least a further 5 d. The cultures of 3T3 cells used were arrested in the G_1/G_0 phase of the cell cycle. The cultures were washed twice with DMEM at 37°C to remove residual serum immediately preceding the experiment. Bovine insulin 26 IU mg^{-1} was obtained from Sigma; EGF was supplied by Dr S. Cohen, and FDGF was isolated and purified from the medium conditioned by SV40 BHK cells as described previously⁷. TPA was obtained from Consolidated Midland Corporation (Brewster, New York). Stock solutions of 10 mg ml^{-1} TPA were made up in acetone and kept at -20°C . Equivalent concentrations of acetone were used in all controls. Insets: The experimental conditions were as described above except that the DMEM/Waymouths medium contained $^3\text{H-TdR}$ ($5 \mu\text{Ci ml}^{-1}$; $0.2 \mu\text{M}$) and the cultures were fixed for autoradiography as described previously¹⁵. The bars represent the percentage of nuclei incorporating the $^3\text{H-TdR}$: a, no addition; b 100 ng ml^{-1} TPA; c, peptide; d, 100 ng ml^{-1} TPA + peptide. The concentrations of insulin, FDGF and EGF were $1 \mu\text{g ml}^{-1}$, $1.8 \mu\text{g ml}^{-1}$ and 10 ng ml^{-1} respectively. Foetal bovine serum produced 79%, 79% and 89% autoradiographically labelled nuclei respectively, in the experiments involving insulin, FDGF and EGF.

factors stimulate DNA synthesis in quiescent cultures of mouse and human cells in completely serum-free medium.

Figure 1 shows that TPA interacts synergistically with insulin, EGF and FDGF in stimulating $^3\text{H-TdR}$ incorporation into acid-precipitable material by quiescent cultures of 3T3 cells. The effects are obtained in entirely synthetic, serum-free medium. The interaction between the tumour promoter and EGF or insulin is particularly striking, because neither of these substances produces a substantial stimulation of DNA synthesis by itself. EGF and TPA reproducibly gave a considerable increase in DNA synthesis in eight independent experiments although the magnitude of the increase varied. The inserts in Fig. 1 show that similar results are obtained when the percentage of labelled nuclei incorporating $^3\text{H-TdR}$ was measured, instead of the total $^3\text{H-TdR}$ incorporation. In the presence of a fixed concentration of insulin, TPA stimulates DNA synthesis in a dose-dependent manner (results not shown). The lowest

Fig. 2 Effect of sequential incubation of TPA and growth-stimulating factors on DNA synthesis in quiescent 3T3 cells. a, Quiescent cultures of 3T3 cells were produced as in Fig. 1. Continuous incubations were washed eight times in DMEM at 37°C before the experiment. Black bar, cultures were incubated continuously in 200 ng ml^{-1} TPA for 40 h; vertical striped bar, cultures were incubated continuously in peptide for 40 h; dotted bar, cultures were incubated for 40 h with 200 ng ml^{-1} TPA and peptide; plain bar, cultures were washed twice with DMEM at 37°C , incubated 20 min with hormone, washed six times in DMEM at 37°C and incubated for 40 h with 200 ng ml^{-1} TPA; oblique striped bar, cultures were washed twice with DMEM at 37°C , incubated 20 min with 200 ng ml^{-1} TPA, washed six times in DMEM then incubated for 40 h with hormone. The final incubation for all cells was in 2 ml 1:1 Waymouths-DMEM containing $^3\text{H-TdR}$ ($0.5 \mu\text{Ci ml}^{-1}$ $0.5 \mu\text{M}$). Incorporation of $^3\text{H-TdR}$ into acid-precipitable material was measured as in Fig. 1. Concentrations of peptides were insulin $1 \mu\text{g ml}^{-1}$, FDGF $0.7 \mu\text{g ml}^{-1}$, EGF 10 ng ml^{-1} . b, Percentage of nuclei labelled autoradiographically after continuous incubation with TPA and insulin (●), TPA or insulin alone (■), or by two washes with DMEM at 37°C followed by 20 min incubation with TPA, six washes of DMEM at 37°C , and then incubation with $1 \mu\text{g ml}^{-1}$ insulin (▲). All continuous incubations were washed eight times at the start of the experiment. The final incubation of all cultures was in 2 ml of 1:1 Waymouths-DMEM containing $^3\text{H-TdR}$ ($5 \mu\text{Ci ml}^{-1}$ $0.2 \mu\text{M}$). All points are in duplicate. Autoradiography was performed as described previously¹⁵. 10% serum produced 86% labelled nuclei at 35 h.



concentration of TPA needed to show synergism consistently with $1 \mu\text{g ml}^{-1}$ insulin was 5 ng ml^{-1} . In contrast to the effect of TPA, phorbol itself, tested either in the absence or presence of insulin, was completely inactive in stimulating DNA synthesis. The close analogue, 4-*O*-methyl-12-*O*-tetradecanoyl-phorbol-13-acetate, was slightly mitogenic (18% of the maximal effect of TPA with insulin) at high concentrations ($1 \mu\text{g ml}^{-1}$) and in the presence of $1 \mu\text{g ml}^{-1}$ insulin.

An interesting feature of the synergistic interaction between TPA with insulin is that a brief exposure (20 min) to the promoter was sufficient to render the cultures sensitive to the mitogenic effect of insulin, EGF or FDGF (Fig. 2a). Although the effect of TPA persisted even after thorough washing of the cell monolayer (six times with DMEM at 37°C), it is not known whether the promoter binds to the cultures in a non-washable manner of whether it causes a lasting change in the culture⁸. Brief exposure of cultures to insulin, EGF or FDGF did not result in their becoming sensitive to the effects of TPA (Fig. 2a).

Table 1 Synergistic interaction between TPA and insulin on DNA synthesis by quiescent cultures of human embryo lung fibroblasts and 3T6 cells

	Cell type	
	Human embryo lung fibroblasts	3T6
No addition	4.2%	1.7%
10 ng ml^{-1} TPA	7.0%	27.0%
100 ng ml^{-1} insulin	3.6%	8.3%
10 ng ml^{-1} TPA + 100 ng ml^{-1} insulin	22.0%	72.0%

Swiss 3T6 cells were maintained in DMEM containing 10% fetal calf serum and subcultured at 3-d intervals. Quiescent cultures in 30-mm dishes were obtained as previously described¹⁶. Experimental conditions for autoradiography were as in Fig. 1. Primary cultures of human lung embryo lung fibroblasts (provided by Dr F. Balkwill), were maintained in 10% fetal calf serum 90% RPMI 1640 and subcultured once every 7 d. For the above experiment, with 7th passage cells, cells were subcultured into 30-mm plates containing RPMI 1640 supplemented with 0.5% foetal calf serum depleted by 7-d incubation with parallel cultures of human embryo fibroblasts, and left for 5 d. They were then washed twice and left in serum-free RPMI 1640 for 4 d. Cells were incubated at 37°C in RPMI 1640 containing ^3H -TdR ($5 \mu\text{Ci ml}^{-1}$ $0.2 \mu\text{M}$) and different additions for 40 h. Autoradiography was carried out as in Fig. 1.

In 3T3 cells, TPA and insulin stimulated DNA synthesis after a lag of approximately 13 h (Fig. 2b). Addition of whole serum stimulated DNA synthesis after a similar lag. The time course of DNA synthesis produced by brief exposure to TPA followed by washing and insulin addition is similar to that produced when insulin and TPA are incubated continuously with the cells (Fig. 2b).

The striking synergistic interaction between growth-promoting polypeptides and TPA was also observed when cell multiplication was monitored over a period of several days (Fig. 3). In these experiments, a certain amount of depleted serum (see legend to Fig. 3) was required to prevent cell detachment. Neither TPA nor insulin added to such cultures caused a substantial increase in growth, however, both insulin and TPA together caused a 2–3-fold increase in cell number. Similarly, although EGF and insulin together caused a two-fold increase in cell number, the addition of the tumour promoter with both insulin and EGF resulted in four-fold increase in cell number.

The synergistic interaction between insulin and tumour promoters was also observed in quiescent cultures of early passage human embryo fibroblasts as well as murine 3T6 cells (Table 1). Insulin potentiated the effect of low concentrations of TPA in both cell types. TPA tested at higher concentrations (100 – 300 ng ml^{-1}) produced a substantial increase in the labelling index of both cell types (results not shown).

The present experiments indicate that there is a striking synergistic interaction between TPA and growth factors in

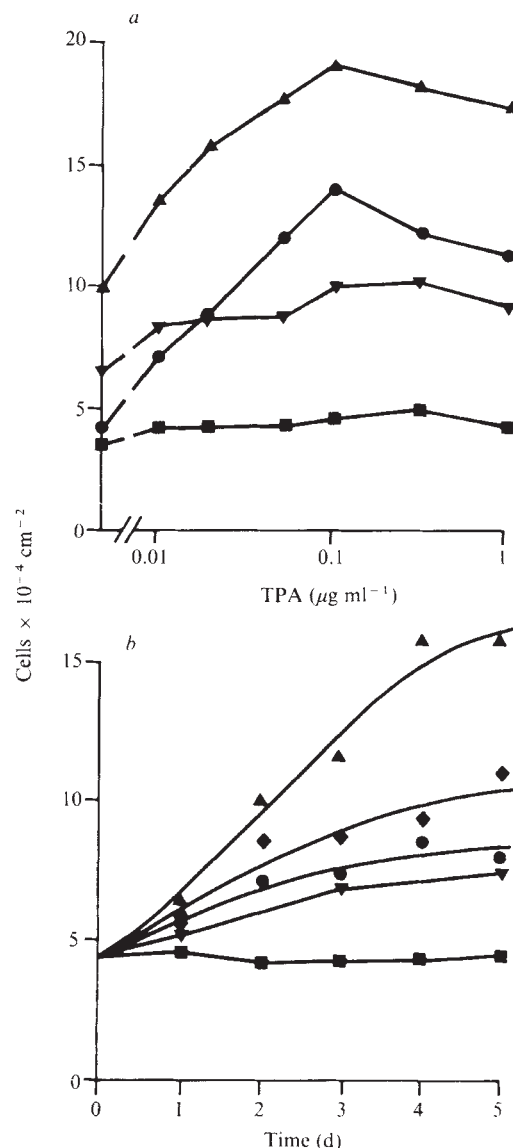


Fig. 3 Effect of insulin, EGF and TPA on cell density when added to quiescent cultures of Swiss 3T3 cells. Culture conditions were as in Fig. 1. Medium from parallel cultures was removed and one-third its volume of Waymouth's medium added to replace low molecular weight nutrients that might have become depleted. TPA, insulin and EGF were then added to give final concentrations as in the figures. Cell number determinations were made on duplicate plates. For each culture, two aliquots of cell suspension, produced by thorough trypsinisation were counted on a Coulter counter. *a*, Effect of different concentrations of TPA on cell density after 5 d of exposure in the absence (■) or in the presence of: (▼) 10 ng ml^{-1} EGF; (●) $1 \mu\text{g ml}^{-1}$ insulin; (▲) 10 ng ml^{-1} EGF and $1 \mu\text{g ml}^{-1}$ insulin. Initial cell density was $4.0 \times 10^4 \text{ cells cm}^{-2}$. *b*, Time course of the effect on cell density produced by addition of: (■) 200 ng ml^{-1} TPA, $1 \mu\text{g ml}^{-1}$ insulin, or 10 ng ml^{-1} EGF alone; (▼) 10 ng ml^{-1} EGF and 200 ng ml^{-1} TPA; (●) $1 \mu\text{g ml}^{-1}$ insulin and 200 ng ml^{-1} TPA; (◆) $1 \mu\text{g ml}^{-1}$ insulin and 10 ng ml^{-1} EGF; (▲) $1 \mu\text{g ml}^{-1}$ insulin, 10 ng ml^{-1} EGF and 200 ng ml^{-1} TPA together. Initial cells density was $4.4 \times 10^4 \text{ cells cm}^{-2}$.

stimulating DNA synthesis in quiescent cultures of mouse and human fibroblasts maintained in serum-free medium. Chemically defined conditions are not only of importance for the study of the mechanism of action of TPA, but may also provide more reproducible assay conditions to screen for tumour promoters or anti-tumour promoters than systems using serum^{2,13}. Our findings raise interesting questions regarding other important cellular effects of phorbol ester. It is now reasonable to ask whether in enhancing the expression of the transformed

phenotype^{9,10}, or in blocking terminal differentiation in a variety of cell types^{11,12}, tumour promoters interact with growth-promoting factors added to the medium, present in serum or produced by the cells. Indeed, the possibility that promotion *in vivo* can be induced by an interaction between the promoter and growth factors should receive serious consideration.

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Direct resorption of bone by human breast cancer cells *in vitro*

OSTEOLYTIC bone metastases occur frequently in patients with advanced malignancy. Breast cancer, the most common malignant disease of women, metastasises to bone more frequently than to any other organ¹, and over 80% of patients with advanced breast cancer develop bone metastases²⁻⁴. The cellular and molecular mechanisms of bone destruction in patients with cancer are not clearly understood. It has been postulated that there may be two mechanisms, one which is mediated by osteoclasts and one which occurs independently of osteoclasts^{5,6}. We show here that cultured human breast cancer cells have the capacity to resorb directly *in vitro*, independently of osteoclast stimulation.

Supernatant media collected from the human breast cancer cell line MCF-7 (ref. 7) were tested for bone-resorbing activity by culture with live and killed fetal rodent long bones previously incorporated with ⁴⁵Ca or ³H-proline. The assay system for live bones has been described in detail previously^{8,9}. The killed bones were devitalised by overnight exposure to UV light to leave a calcified acellular bone matrix. There is no glucose uptake by these bones and they do not respond to parathormone (PTH) or prostaglandins. Killing the bones allows assessment of the effect of exogenous cells on bone resorption independently of the effects of endogenous bone cells¹⁰. The bones were labelled with ⁴⁵Ca or ³H-proline¹¹ by injection of the mothers 1-3 d before death. Mineral release and matrix resorption were assessed in the same way as for live bones. Experiments on matrix resorption were carried out with mouse bones rather than fetal rat bones because it was easier to incorporate the label into the former, probably because of the smaller volume of distribution of the isotope in the pregnant mice.

Table 1 shows that in three separate experiments supernatants from MCF-7 cell cultures caused the release of previously incorporated ⁴⁵Ca from live and dead fetal rat long

Table 1 Effects of supernatant media from cultured human breast cancer cells (MCF-7) on mineral release from live and dead fetal rat long bones compared with paired bones treated with control media

Bones	⁴⁵ Ca Release (treated/control ratios)		
	Expt A	Expt B	Expt C
Live	1.39 ± 0.13*	1.75 ± 0.06*	1.96 ± 0.10*
Dead	1.59 ± 0.06*	1.41 ± 0.09*	1.77 ± 0.03*

MCF-7 cells were grown in monolayer culture in Dulbecco's modified Eagle medium (Gibco) with 10% heat-inactivated fetal calf serum. Cell-free supernatants were collected from cultures which were near confluency. The assay for bone resorption is based on the release of previously incorporated ⁴⁵Ca from fetal rodent long bones in organ culture^{8,9}. Bone-resorbing activity was quantitated as the ratio of ⁴⁵Ca released into the media by test bones compared with ⁴⁵Ca released into the media from paired bones cultured in control media. Each bone was cultured for 48 h with 0.5 ml of media from the MCF-7 cultures. Values are means ± s.e.m. for 4 pairs of bone cultures.

*Significantly greater than 1.0, $P < 0.05$.

bones. Similar results were obtained when the bones were co-cultured with the MCF-7 cells. Table 2 shows that the bone matrix was also resorbed by the MCF-7 cells and their conditioned media. In these experiments we determined the proportion of the radioactivity released into the media from the bones which was in the form of ³H-hydroxyproline, because this latter component reflects bone collagen breakdown¹¹. Media from individual bone cultures were pooled before chromatography on a Dowex column so that standard errors could not be calculated. There was very close agreement between the data obtained from release of total radioactivity and the data obtained from release of ³H-hydroxyproline (Table 2).

Table 3 shows that the supernatants from four other established breast cancer cell lines caused mineral release from devitalised fetal rat bones. Other normal and neoplastic cells had no effect on bone mineral release. The effects of MCF-7 cells on bone could not be ascribed to changes in pH. Resorption occurred in experiments where there was no change in the measured pH of the media, and also when the pH of the supernatant media was readjusted to 7.4 before the bones were cultured in the cell-free media.

Fig. 1 Effects of pharmacological inhibitors of osteoclast activity on bone resorption due to human breast cancer cells (MCF-7) and prostaglandin-E₂ (10⁻⁷ M). The bone-resorbing effects of the MCF-7 supernatants were not altered by cortisol (10⁻⁶ M) or phosphate (6 mM). In contrast, PGE₂-mediated bone resorption was inhibited by these drugs. Bone resorption is measured as per cent increase in ⁴⁵Ca release from bones cultured with MCF-7 culture supernatants or PGE₂ compared with paired bones cultured in identical media except for the MCF-7 supernatant or PGE₂. a, MCF-7; b, MCF-7+phosphate; c, MCF-7+cortisol; d, PGE₂; e, PGE₂+phosphate; f, PGE₂+cortisol.

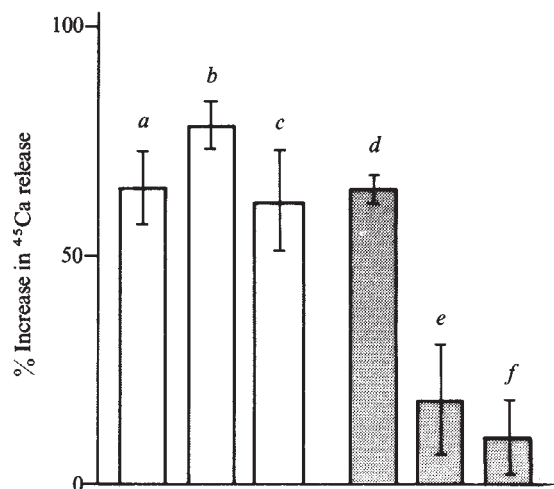


Table 2 Effects of cultured human breast cancer cells (MCF-7) on matrix resorption of live and dead fetal rodent long bones labelled with ^3H -proline

Bones	Total radioactivity released (treated/control ratios)*	^3H -hydroxyproline released (treated/control ratios)
Live		
MCF-7 supernatants	$1.67 \pm 0.10^\dagger$	1.65
PTH (400 ng ml $^{-1}$)	$1.74 \pm 0.04^\dagger$	1.75
Dead		
MCF-7 cells	$2.26 \pm 0.26^\dagger$	2.0
MCF-7 supernatants	$1.38 \pm 0.09^\dagger$	1.24

Fetal bones were labelled with ^3H -proline by injection of the mother 2 d and 1 d before death. Each bone was cultured with 0.6 ml media. At the end of the culture period, 100- μl aliquots of this media were removed and counted in a liquid scintillation counter so that treated-to-control ratios for total radioactivity released from pairs of bones could be obtained. The remaining media for each treatment group were pooled and applied to a Dowex column to separate ^3H -hydroxyproline from ^3H -proline²⁰. All the radioactivity applied to this column was recovered in the fractions corresponding to where markers of proline and hydroxyproline were eluted. The duration of the bone cultures was 2 d. In some dishes, MCF-7 cells were co-cultured with the bones and in others supernatants taken from MCF-7 cultures near confluency were used.

*Values are means $\pm$ s.e.m. for 4–12 pairs of bone cultures.

† Significantly greater than 1.0, $P < 0.05$.

Morphological features of resorbing live bones which were cultured with MCF-7 media were examined and compared with bones cultured with prostaglandin- E_2 (10^{-6}M). The bones which were cultured with prostaglandins showed many giant multinucleated osteoclasts adjacent to the endosteal bone surfaces. These bones were resorbed 80% more than corresponding paired control bones as measured by ^{45}Ca release. Similar features are found when bones are cultured with other humoral stimulators such as PTH, osteoclast-activating factor, active vitamin D metabolites¹², and thyroid hormones¹³. In contrast, bones which were cultured with supernatants from the MCF-7 cultures showed no detectable osteoclasts although there was a similar release of ^{45}Ca (83% increase in ^{45}Ca release compared with corresponding control).

To confirm that osteoclasts are not required for breast cancer cells to resorb bone, live bones were cultured with cortisol (10^{-6}M) and phosphate ($6 \times 10^{-3}\text{M}$), both of which inhibit the

effects of osteoclasts on bone^{14,15}. Phosphate and cortisol had no effect on bones resorbing in response to MCF-7 media (Fig. 1). On the other hand, prostaglandin-stimulated bone resorption was inhibited in bone cultures to which cortisol or phosphate were also added. These data also indicate that the MCF-7 media resorbed bone independently of osteoclasts.

Prostaglandins could not have played any part in the effects that we have observed. They do not resorb devitalised bone¹⁶, and phosphate and cortisol, which inhibited prostaglandin-stimulated bone resorption, had no effect on MCF-7 supernatants (Fig. 1). We confirmed that bone resorption stimulated by MCF-7 cells occurred independently of prostaglandins by culturing MCF-7 cells with indomethacin (10^{-5}M) on live bones. Indomethacin, which inhibits prostaglandin synthesis at this concentration, had no effect on the release of ^{45}Ca from these live bones resorbed by the MCF-7 cells (data not shown). The conditioned media from MCF-7 cells do not contain sufficient immunoreactive prostaglandins of the E or $\text{F}_{2\alpha}$ types to cause bone resorption in this assay (data not shown).

Although our data show no evidence of osteoclast stimulation by breast cancer cells *in vitro*, this does not preclude a role for osteoclasts in metastasis formation and hypercalcaemia *in vivo*. It is possible that osteoclasts could be stimulated at metastatic sites by factors released by cells other than cancer cells. Local bone-resorbing factors such as prostaglandins¹⁶ or osteoclast-activating factor¹⁷ could be released by monocytes¹⁸, activated lymphocytes¹⁷ or fibroblasts as part of a cell-mediated immune response to the tumour. These effects would not be observed in our experiments.

Every breast cancer cell line that we tested resorbed bone *in vitro* (Table 3). This correlates with the clinical observation that nearly all patients with breast cancer will develop bone metastases if they live long enough with the disease²⁻⁴. Not all cultured neoplastic cells have this property (Table 3), but more work will be required to determine how frequently tumour cells in culture behave in this way.

Thus, these *in vitro* results suggest that there may be at least two mechanisms for the bone destruction which occurs in patients with malignant disease. Bone destruction may be osteoclast mediated and this is the probable mechanism in patients with myeloma¹⁹ and with solid tumours who develop ectopic hormone syndromes. However, the studies described here show that some cancer cells can resorb bone directly independently of osteoclasts. In some patients it is possible that both mechanisms are relevant^{5,6}.

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Table 3 Effects of cell culture supernatants on mineral release from devitalised fetal rat long bones compared with paired bones cultured with control media

Cell line	Source	Clinical data	^{45}Ca release (treated/control ratios)
MCF-7	Malignant pleural effusion	Not known	$1.42 \pm 0.07^*$
MDA-MB-157	Malignant pleural effusion	Not known	$1.34 \pm 0.08^*$
MDA-MB-231	Malignant pleural effusion	Not known	$1.54 \pm 0.13^*$
MDA-MB-330	Malignant pleural effusion	Bone metastases	$1.43 \pm 0.09^*$
MDA-MB-134	Malignant pleural effusion	Bone metastases	$1.35 \pm 0.13^*$
3T3 Cells	Mouse fibroblasts	No abnormality	1.08 ± 0.05
Normal lymphocytes	Human peripheral blood	No abnormality	1.05 ± 0.03
Chronic lymphocytic leukaemia lymphocytes	Human peripheral blood	Osteopenia, normal serum calcium	0.96 ± 0.03
Ovarian cancer	Human primary tumour	Hypercalcaemia, no bone metastases	0.99 ± 0.05
Lung cancer	Human primary tumour	Hypercalcaemia, no bone metastases	0.90 ± 0.12
Chondroblastoma	Human primary tumour	No skeletal abnormality	1.02 ± 0.03

The duration of the bone cultures was 2 d. Supernatants were taken from tumour cells and 3T3 cells which were near confluency. The non-adherent lymphocytes were cultured for 2 d at a cell density of 1.5×10^6 cells ml $^{-1}$ in Dulbecco's modified Eagle medium and 10% fetal calf serum. MCF-7, MDA-MB-157, MDA-MB-231, MDA-MB-330 and MDA-MB-134 are established human breast cancer cell lines. Values are means $\pm$ s.e.m. for 4 pairs of bone cultures.

*Significantly greater than 1.0, $P < 0.05$.

cancer cell lines were obtained from E. G. and G. Mason Research Institute.

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Selective expression of transduced or cloned DNA in minicells containing plasmid pKB280

DERIVATIVES of coliphage λ , carrying inserts of essentially any prokaryotic or eukaryotic DNA, can now be constructed using novel λ cloning vectors. Often, the reason for constructing these derivatives is to determine the polypeptides encoded by the inserted DNA. Phage expression can be analysed by infection of anucleate *Escherichia coli* minicells in which all host syntheses are absent¹. However, analysis of the expression of the non- λ DNA insert in this system is complicated by the concurrent expression of λ DNA. Attempts to inhibit the expression of the λ genes by use of minicells from a λ lysogenic minicell-producing strain were unsuccessful, indicating that insufficient λ repressor was segregated into the minicells (Shaw, Epp, Pearson and J.N.R., in preparation). Recently a plasmid, pKB280, was constructed which carries the gene (*cI*) for the λ repressor protein and in which approximately 1% of the soluble protein of *E. coli* strains carrying this plasmid is λ repressor². Minicells produced by a derivative of *E. coli* DS410 carrying plasmid pKB280 have been infected by transducing and cloning phages. We report here that only the non- λ DNA is expressed in these minicells.

E. coli DS410 (pKB280) was constructed by transformation of *E. coli* DS410 with purified plasmid DNA and selection for tetracycline resistance and immunity to λ . The plasmid would be expected to segregate into minicells, so that minicells produced by this strain should synthesise plasmid-encoded polypeptides. Figure 1 shows that minicells from *E. coli* DS410 (pKB280) synthesise large amounts of a polypeptide of molecular weight 26,000, presumably λ repressor, whose molecular weight from amino-acid sequence data is 26,228 (ref. 4). In addition, a further 24 polypeptides are detectable which, together with repressor, would require a total coding capacity of 10,700 base pairs of DNA. Plasmid pKB280 is approximately 6,800 base pairs in length^{2,5}. It is clear, therefore, that the observed polypeptides exceed the coding capacity of the plasmid. As all the polypeptides synthesised are smaller than the repressor poly-

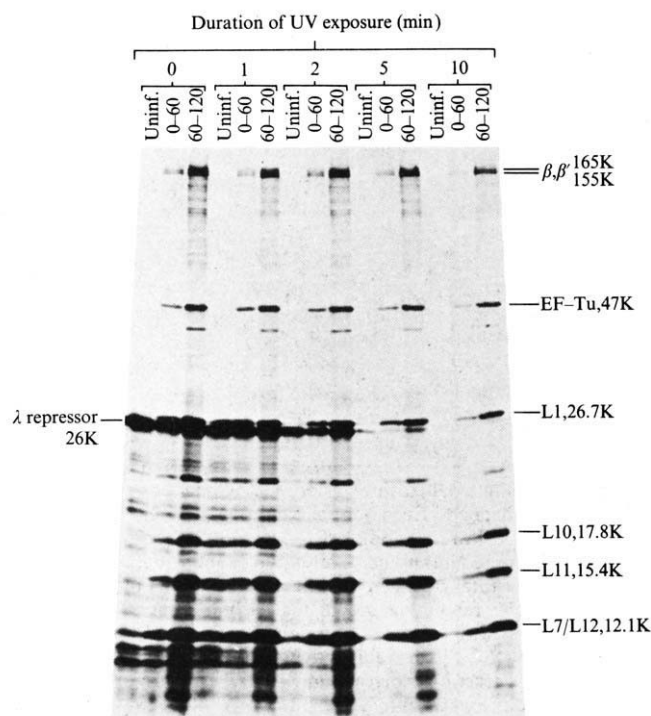


Fig. 1 Autoradiogram of the electrophoretic separation of the polypeptides synthesised in uninfected and λ rif^d18-infected minicells of *E. coli* DS410 (pKB280). Minicells were prepared as previously described and suspended (2×10^{10} per ml) in M9 minimal salts medium containing $20 \mu\text{g ml}^{-1}$ D-cycloserine^{1,3}. The minicells were exposed to ultraviolet irradiation ($290 \mu\text{W cm}^{-2}$) for the indicated times. Aliquots of the infected minicells were infected (MOI = 5) with λ rif^d18 phage. Uninfected and infected minicells were incubated at 37°C in the presence of ^{35}S -methionine assay medium^{1,3}. The uninfected minicells and one aliquot of infected minicells (0-60) were allowed to incorporate the radioactivity for 1 h. A second aliquot of infected minicells was incubated in M9 glucose-salts minimal medium for 1 h at 37°C before addition of ^{35}S -methionine assay medium. These samples were then allowed to incorporate radioactivity for 1 h (60-120). Preparation of the radioactively labelled polypeptides synthesised in the minicells for electrophoresis, polyacrylamide gel electrophoresis through 14-20% polyacrylamide gradient gels, impregnation for fluorimetry and production of autoradiograms have been described previously^{1,3}.

peptide, it seems likely that incomplete forms of repressor and/or proteolytic cleavage of the repressor occur in minicells.

Infection of the minicells by λ rif^d18 was used to test the function of the λ repressor in minicells. Phage λ rif^d18 carries the genes for β and β' subunits of RNA polymerase, for EF-Tu and for the ribosomal proteins L1, L10, L11 and L7/L12 (refs 6-9). Infection results in the synthesis of only non- λ polypeptides encoded by λ rif^d18 in addition to the plasmid-encoded polypeptides (Fig. 1). No λ genes are expressed, indicating that the repressor inhibits the expression of the λ portion of λ rif^d18. The minicells were exposed to ultraviolet irradiation before infection, to destroy the synthesising ability of the plasmid (Fig. 1), and subsequent infection by λ rif^d18 resulted in the synthesis of only the non- λ polypeptides encoded by the phage genome.

Infection of the repressor-containing minicells by λ wt phage results in the synthesis of reduced amounts of only two b2 region non-essential gene products (pEa47 and pEa59 in Fig. 2a). These two polypeptides have also been found to be synthesised in infected minicells in the absence of λ N protein (Shaw and J.N.R., unpublished observation). Fortuitously, the genes encoding these polypeptides are usually deleted in transducing or cloning phages, as in the phage λ dgal70/4 (ref. 10) shown in Fig. 2b. Infection by λ dgal70/4 of minicells containing repressor results in the synthesis of only the *gal* operon polypeptides.

There are basically two types of λ -cloning vectors. First, the insertion type in which only one restriction endonuclease site

remains in the vector DNA and which is often located in the *cI* gene itself. Second, replacement vectors which have two restriction sites flanking a replaceable fragment of DNA¹¹. Figure 2b shows the result of expression in minicells of an insertion vector (NM607 (ref. 11)) or replacement vector (NM596) which carry the same fragment of cloned DNA, namely the 4.7 megadalton fragment of DNA derived from plasmid VH151 (refs 12, 13) which encodes the *trpDEop* region of the *E. coli* chromosome. The *trpD* and *trpE* gene products can be readily identified from their published molecular weights¹⁴ (Fig. 2b). Expression of the λ genes of the replacement vector is inhibited by the high concentration of λ repressor in the plasmid-containing minicells. Only the *trpD* and *trpE* gene products and a polypeptide of molecular weight 37,000 are synthesised in the presence of λ repressor. The 37,000 polypeptide may result from expression of the fusion of part of the *trpC* gene which is also carried on the cloned fragment¹³ with the adjoining λ DNA. In contrast to the replacement vector, however, even in the presence of high concentrations of λ repressor, infection of minicells by the phage with the *trpDEop* fragment inserted in the *cI* gene results in the synthesis of polypeptides encoded by both the λ and the cloned DNA. This is exactly what would be expected, because the *cI* gene of this insertion vector, as found for many insertion vectors¹¹, is not the homologous λ *cI* gene but rather the *cI* gene of the lambdoid heteroimmune phage 434 (ref. 11). Expression is therefore insensitive to λ repressor. A plasmid carrying the immunity region of phage 434 has recently been isolated (A. Bailone and R. Devoret, personal com-

munication). Construction of a minicell strain carrying this plasmid should provide a system in which DNA cloned in *i*⁴³⁴ phages can be selectively expressed.

In conclusion, it seems possible to use ultraviolet-irradiated minicells produced by *E. coli* DS410 (pKB280) to express exclusively cloned or transduced DNA *in vivo*. The expression requires that the fragment of DNA of interest carries a promoter which is insensitive to λ repressor. Expression of fragments of DNA which do not carry promoters may still be analysed in minicells without λ repressor; however, λ expression will also occur¹. Comparison of the polypeptides synthesised in minicells containing or lacking repressor infected by vector phage with and without cloned or transduced DNA should enable the following questions to be answered. (1) Are genes on the fragment of DNA expressed in *E. coli*? (2) Does the fragment of DNA carry a promoter which functions in *E. coli*? (3) If (1) is positive, then how many and what are the molecular weights of the encoded polypeptides (or mRNAs), and if (2) is positive, can the expression be affected by the experimental conditions, such as an inducing compared with a non-inducing medium?

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Note added in proof: Minicells from *E. coli* DS410 carrying the plasmid pGY101 (from R. Devoret) which confers immunity to *i*⁴³⁴ phages have been found to function as predicted and permit the selective expression of non- λ DNA contained in *i*⁴³⁴ vector genomes.

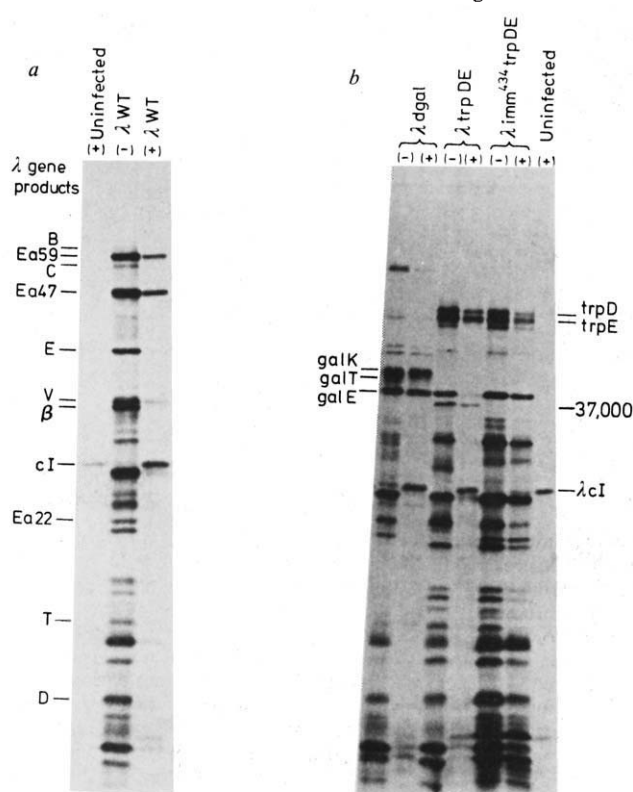
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Fig. 2 Comparison of the polypeptides synthesised in infected minicells in the presence and absence of λ repressor. Minicells were prepared from *E. coli* DS410 (–) and *E. coli* DS410 (pKB280) (+). The minicells were UV irradiated for 8 min (290 μ W cm^{–2}) at a concentration of 2×10^{10} minicells per ml. **a**, Minicells infected by λ wt (λ cI₈₅₇S_{am7}); **b**, minicells infected by λ dg_{al} 70/4 (ref. 10); λ replacement vector NM596, nin 5, carrying the *trpDEop* fragment of the *E. coli* chromosome^{12,13} between *Eco*RI sites 2 and 3 of λ ; λ insertion vector NM607, b₅₃₈, carrying the *trpDEop* fragment of the *E. coli* chromosome inserted in the *Eco*RI site located in the *imm*⁴³⁴ *cI* gene¹¹. Infected and uninfected minicells were allowed to incorporate ³⁵S-methionine for 2 h at 37°C in M9 minimal salts medium. Electrophoretic and other methods were as described in Fig. 1.



Assembly of stratum corneum basic protein and keratin filaments in macrofibrils

THE stratum corneum is the most superficial aspect of mammalian epidermis and consists of several layers of fully differentiated, dead cells filled with an insoluble protein complex called keratin. Early ultrastructural studies of the stratum corneum cells revealed a 'keratin pattern' of light-staining filaments 70–80 Å in diameter embedded in a dark-staining osmiophilic amorphous matrix¹. The filaments are synthesised in the inner living cell layers and are variously termed tonofilaments, α -keratin or, more accurately, keratin filaments. The subunits of the filaments of both the living cells and stratum corneum have been extracted with dissociating reagents from bovine^{2–4}, rat^{5–6}, mouse⁷, and human⁸ epidermis. In appropriate conditions, these subunits will polymerise *in vitro* into native-type keratin

filaments that are uniformly 70–80 Å in diameter^{7,9}. In contrast, the amorphous matrix has not been positively identified at the biochemical level, although it was recently suggested that a stratum corneum basic protein (SCBP) rich in histidine has properties consistent with a possible function as an interfilamentous matrix substance¹⁰. We show here that highly ordered structures reminiscent of the keratin pattern in the intact stratum corneum form when the SCBP and epidermal keratin filaments are combined *in vitro*.

SCBP was purified from newborn rat epidermis as previously described¹⁰. The keratin filament subunits were isolated from the same source by a modification of previous techniques^{3,5}. Other fibrous proteins were also combined with SCBP in the aggregation experiments. These included: keratin filaments assembled *in vitro* from the subunits extracted from mouse⁷, human and bovine⁹ epidermis; β -keratin filaments polymerised *in vitro* from the *S*-carboxymethyl derivatives of chicken feather¹¹; mouse skin collagen, types I and IV (ref. 12); F-actin isolated from mouse skeletal muscle and mouse epidermal cells grown in culture¹³.

The rat epidermal keratin filaments outlined by negative-staining with uranyl acetate are apparently identical to those of other species^{7,9}. They are long rods uniformly 70–80 Å in diameter, but in places display a distinct electron-dense central line suggestive of a core (Fig. 1a).

When SCBP was combined with these filaments in ratios as low as 0.1:1 (by weight), there was an immediate increase in turbidity indicating association of the proteins. At ratios of 0.2:1 or greater, easily observable fibrous-like aggregates precipitated from suspension. The amount of aggregate increased with increasing ratios of SCBP to filaments up to about 2:1. Under the electron microscope, these aggregates were composed of large numbers of orientated keratin filaments (Fig.

1b,c). Bundles of filaments in differentiating keratinocytes are termed macrofibrils¹⁶, a term which might also accurately describe these aggregates formed *in vitro*. When formed at low SCBP-to-filament ratios, filaments in the macrofibrils assembled in either a loose or tight organisation but when formed at higher ratios, more compact macrofibrils generally resulted in which filaments were highly aligned and tightly packed. The macrofibrils formed within seconds of mixing and were stable for several days at room temperature and indefinitely at 4°C.

Samples of the macrofibrils were fixed, embedded, sectioned and observed in both longitudinal and cross section. In longitudinal section, lightly stained filaments 70–80 Å in diameter were aligned in close apposition and were separated at an approximately uniform distance by dark-staining material (Fig. 2b). In cross section, darkly stained dots were observed surrounded by light haloes. These structures corresponded to the central dense core and the electron-lucent portions of the filament. The diameter of the light halo is identical to the diameter of the filaments seen in longitudinal sections. The filaments, in turn, were outlined by darker-staining material which filled the interfilamentous interstices (Fig. 2c). This pattern closely resembles the keratin pattern of certain stratum corneum cells.

Two types of control experiments were performed. Cytochrome *c* and a histone preparation (calf thymus, type II, Sigma) were each tested as alternate basic proteins. When mixed with rat keratin filaments, there was a slight increase in turbidity indicating association of the basic proteins with the anionic filaments, but no macrofibrils formed. The individual filaments were coated with an amorphous layer of protein and, as a result, seemed irregular in diameter. Other filament preparations were also tested. β -Keratin filaments from chicken feather, mouse dermal collagen types I and IV and F-actin from mouse skeletal

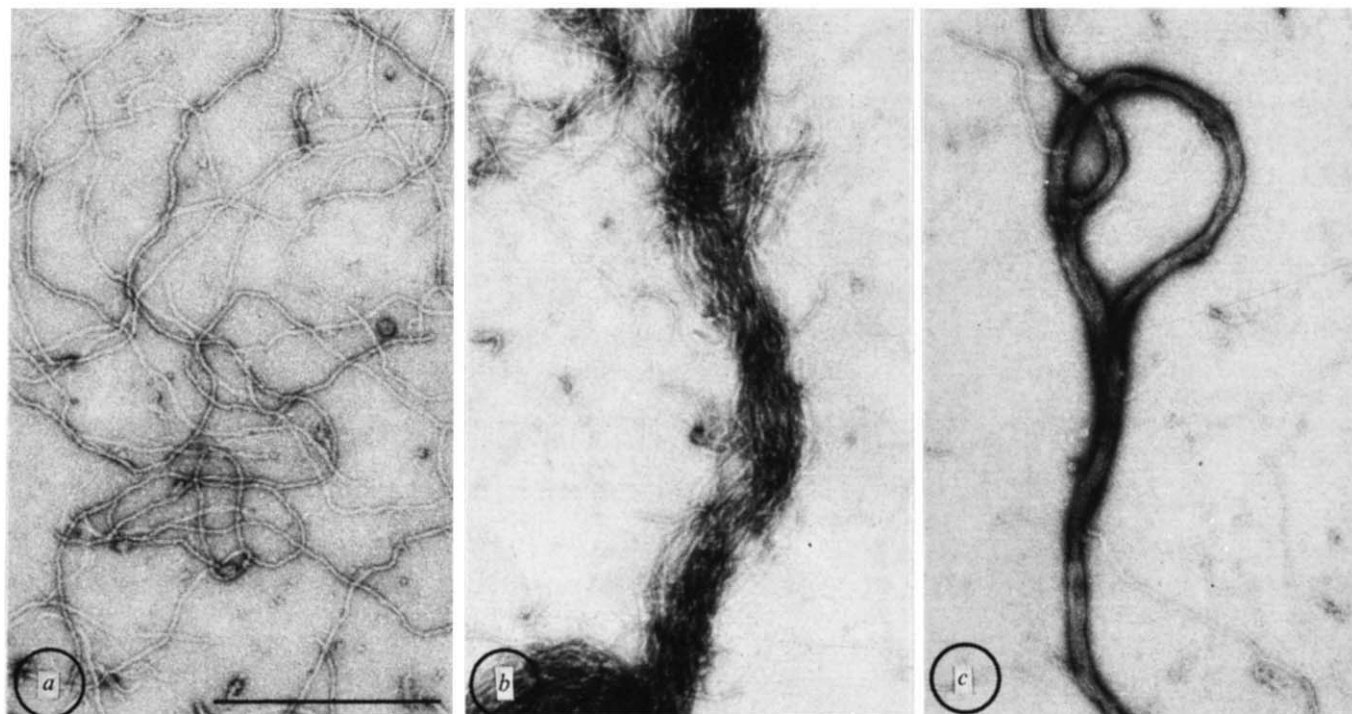


Fig. 1 a, Rat epidermal keratin filaments. Newborn rat epidermis (Sprague-Dawley, Berkeley strain) was separated from dermis and extracted first with 1 M potassium phosphate (pH 7.0) to remove keratohyalin⁴, then with 4 M urea in 0.05 M phosphate (pH 7.0) for 3 h at 23 °C and with 8 M urea in 0.1 M Tris-HCl (pH 8.5) containing 0.1 M 2-mercaptoethanol for 16 h at 23 °C. All extraction buffers contained the protease inhibitor phenylmethylsulphonyl fluoride. The 4 M urea extract was used to prepare SCBP¹⁰, and the 8 M urea extract was the source of the keratin filament subunits. After further purification by DEAE-cellulose chromatography¹⁵, these subunits (1 mg ml⁻¹) were dialysed against 5 mM Tris-HCl (pH 7.6) containing 1 mM dithiothreitol or 25 mM 2-mercaptoethanol for 16 h at 23 °C during which time filaments formed in suspension. For negative staining, filament suspensions were diluted to approximately 0.1 mg ml⁻¹, placed drop by drop onto parlodion and carbon-coated grids, stained with 1% uranyl acetate, dried and examined in a Philips 201 transmission electron microscope (TEM). b and c, recombination of rat epidermal keratin filaments with SCBP. SCBP and filaments were equilibrated at 1 mg ml⁻¹ in 5 mM Tris-HCl (pH 7.6). Samples of the two solutions were mixed at an SCBP-to-filament ratio of approximately 0.25:1 (by weight), allowed to stand for 5 min, placed drop by drop on grids, negatively stained with uranyl acetate and examined by TEM. b, Loose rope-like aggregates of aligned filaments; c, compact macrofibrils with highly aligned filaments. Scale bar, 0.5 μ m.

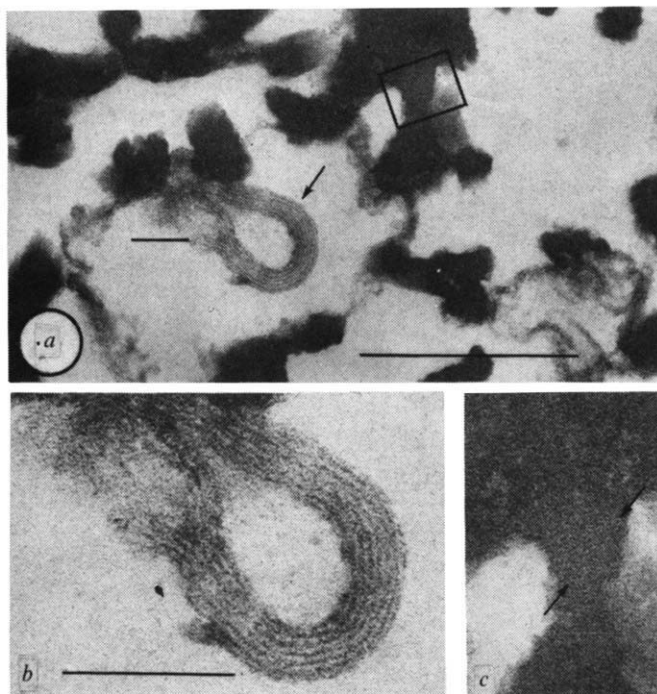


Fig. 2 Ultrastructure of fixed, embedded macrofibrils formed *in vitro* at a ratio of SCBP to filaments of approximately 4:1. Macrofibrils were withdrawn from the tube, fixed for 1 h in half-strength Karnovsky fixative¹⁷, dehydrated through a graded series of alcohols, and embedded in Epon¹⁸. *a*, Filaments seem to be combined with sparse (arrow) and abundant (box) quantities of SCBP. Scale bar, 0.5 μ m. *b*, Aligned filaments in longitudinal section sparsely coated with SCBP. Scale bar, 0.2 μ m. *c*, Cross-sectional view of filaments in a large amount of SCBP. Arrows indicate the black dot of the central dense line within the filament. Same magnification as (*b*).

or cultured mouse epidermal cells did not aggregate into fibres or form macrofibrils when mixed with SCBP. In each case, an increase in turbidity was seen on mixing and the fibrous protein was coated in an apparently random manner by the SCBP.

Thus the formation of macrofibrils *in vitro* is specific for SCBP and epidermal α -keratin filaments. This specificity, the highly orientated nature of these structures, and their close resemblance to the structure seen in intact stratum corneum, lead us to conclude that the SCBP may be an interfibrillar or matrix protein of epidermal keratin.

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Actin microcrystals and tubes formed in the presence of gadolinium ions

SKELETAL muscle actin can be isolated either as a globular monomer, G-actin, or as a filamentous polymer, F-actin^{1,2}. The transition of G to F-actin is achieved by the addition of salts of monovalent or divalent cations. In the presence of higher concentrations of divalent cations F-actin filaments further

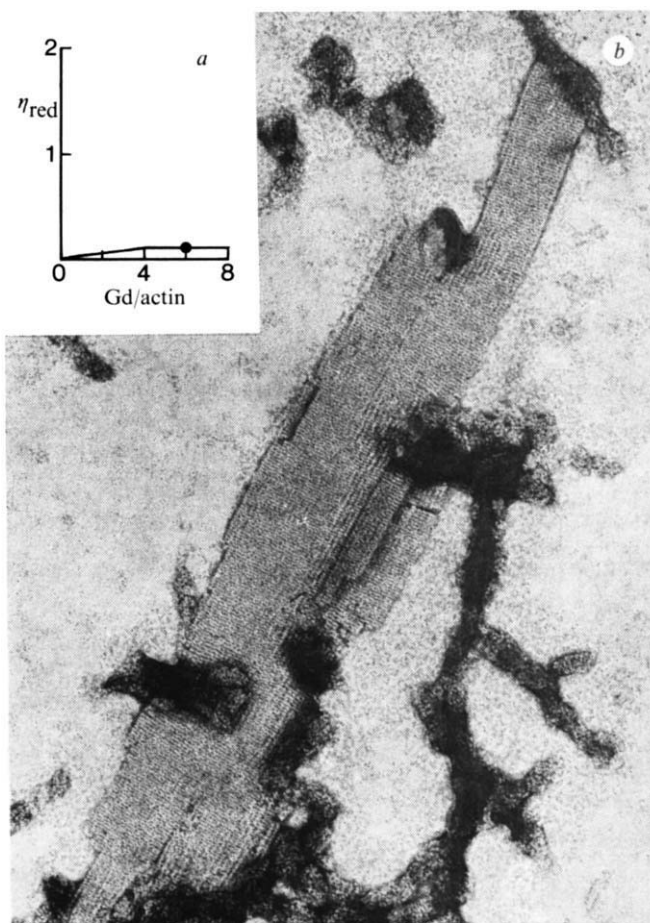


Fig. 1 *a*, Effect of Gd III on the reduced viscosity of actin in the absence of KCl. Reaction conditions are: 0.2 mM ATP; 0.5 mM β -mercaptoethanol; 2 mM PIPES at pH 6.95; 25 °C; GdCl₃ added at the molar ratios indicated on the abscissa. As Gd III binds to ATP the amount stated was that in excess of the ATP present^{8,9}. 1.15 mg ml⁻¹ G-actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt¹⁰. Steady state $\eta_{red} = [(ts/tw) - 1]/C$ ml mg⁻¹: where *ts* is the flow time (s) of the sample, *tw* is the flow time of the solvent (31.5 s) and *C* is the G-actin concentration (mg ml⁻¹) as determined by the Lowry method¹¹. *b*, Actin microcrystal formed with Gd III in the conditions specified by the black dot in (*a*). The overall dimensions of the polymer are about 1 μ m $\times$ 0.2 μ m. In some experiments microcrystals were formed by adding Gd III slowly by dialysis. Specimens were supported on thin carbon-coated copper grids and negatively stained with 1% uranyl acetate. Magnification was calibrated with the known 14.4-nm repeat of thick filaments of the oyster adductor muscle¹². $\times$ 143,000.

aggregate into paracrystalline arrays³⁻⁶. We present here evidence for two new types of ordered actin assembly formed in the presence of trivalent lanthanide cations, which are not composed of F-actin filaments.

Recently we have studied the effects of trivalent lanthanides on the viscosity of G-actin in the absence⁷ and presence⁸ of 0.1 M KCl. These data suggest that amounts of lanthanide ions greater than 5.5 mols per mol of actin cause actin to aggregate into a form different from F-actin. We have now investigated in the electron microscope the aggregates induced by the trivalent lanthanide gadolinium (Gd III). Figure 1*b* shows that without added KCl, Gd III ions cause G-actin to assemble into sheet-like structures which we will refer to as actin microcrystals. These polymers are formed at molar ratios higher than 4 mols Gd III per mol of G-actin. Their dimensions vary but they are usually not longer than 1 μm and not wider than 0.3 μm . There are often sharp steps in the staining density across the structure which suggests that they consist of more than one layer. The microcrystal in Fig. 1*b* is at least three layers thick. They have a prominent axial period of approximately 3.3 nm which is best observed when micrographs are viewed obliquely from the side. A less clear transverse repeat of about 6.0 nm is occasionally seen when the structures are viewed obliquely along their length but this was difficult to measure accurately. Microcrystals differ markedly in appearance from actin paracrystals. In particular they do not show the 36-nm axial repeat characteristic of the long pitched helical repeat of F-actin^{3,4}. Some less well-ordered rope-like aggregates of actin can also be seen in Fig. 1*b*, but no individual filaments of F-actin were present in these preparations.

G-actin forms different polymers when incubation with Gd III ions is followed by the addition of 0.1 M KCl. Figure 2*a* summarises our previously reported data on the changes in viscosity in these conditions^{7,8}. Figure 2*b* shows an example of the polymers formed in the presence of KCl at a molar ratio of

Fig. 2 *a*, Effect of Gd III on the reduced viscosity of actin in the presence of KCl. Reaction conditions are the same as described in Fig. 1*a* except that following a 5 min incubation of G-actin with Gd III, KCl was added to give a final concentration of 0.1 M. *b*, An actin tube formed with Gd III in conditions specified by the black dot in 2*a*. The arrow indicates an end of a tube where only one side of the structure can be seen. Negatively stained with 1% uranyl acetate. $\times 134,000$.

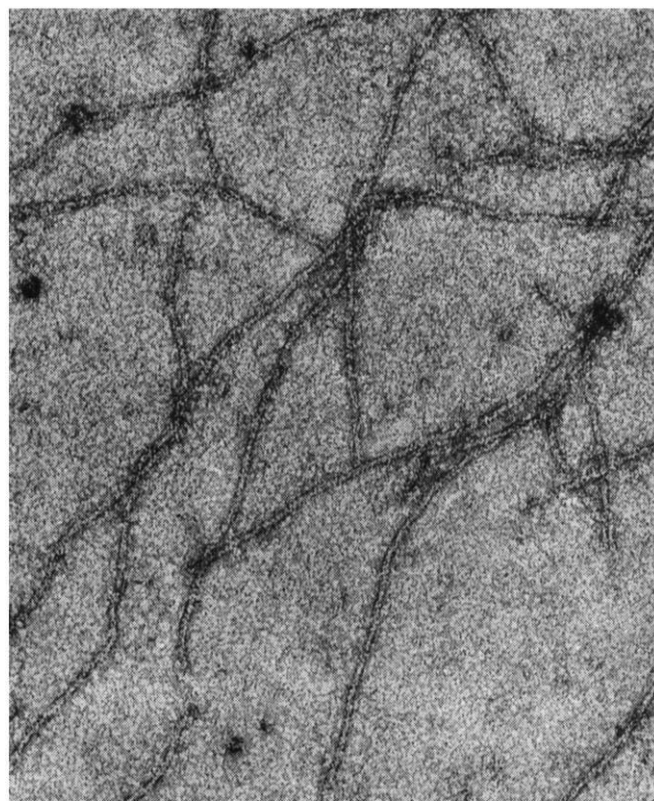
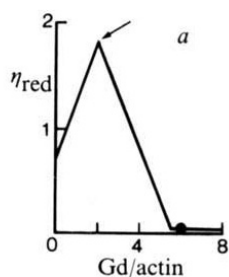
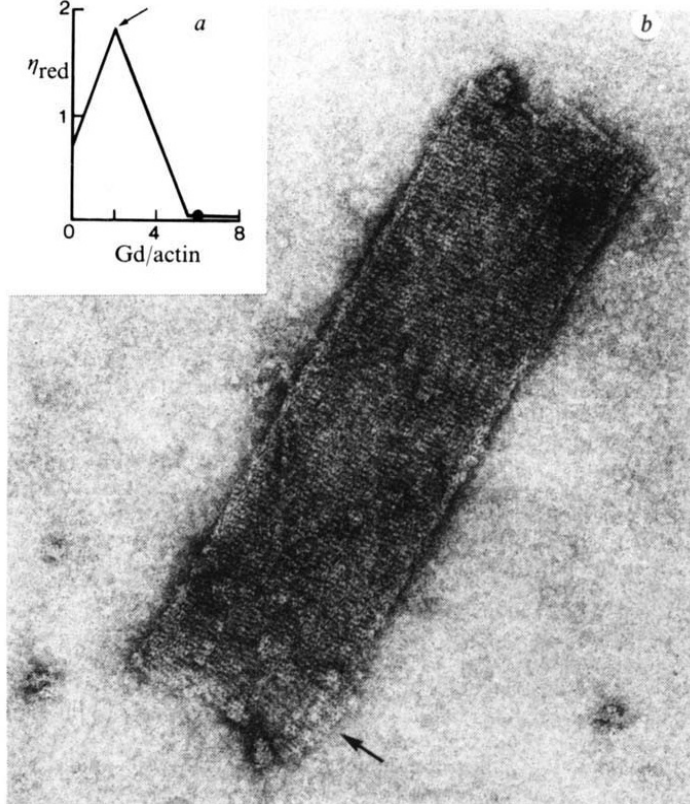


Fig. 3 Network of F-actin formed at the peak of the reduced viscosity in the conditions indicated by the arrow in Fig. 2*a*. No actin tubes were observed in these conditions stained with 1% uranyl acetate. $\times 126,000$.

6 mols or more of Gd III per mol actin. The length of the polymers is variable from ~ 0.3 to $\sim 1.5 \mu\text{m}$ but each polymer has a uniform width of about $200 \text{ nm} \pm 10 \text{ nm}$. In each polymer two sets of diagonal striations spaced about 5.5 nm apart can be seen intersecting one another at equal and opposite angles of approximately 80° with respect to the sides of the structure. This is seen more clearly when the micrograph is viewed at an oblique angle. Occasionally at the ends of the polymer only one set of striations is observed and here the 5.5-nm repeat is distinctly seen. These observations suggest that the main part of the polymer is a flattened tube with the two sets of striations coming from strands that form the front and back of the polymer. We therefore propose to call these structures actin tubes. As in the microcrystals, no 36-nm repeat and no F-actin filaments can be seen in these preparations. Rope-like aggregates which have a similar appearance to the extra material seen with microcrystals are sometimes seen associated with actin tubes. This extra material may be associated with the formation of both structures.

We have shown (Fig. 2) that in the presence of 0.1 M KCl actin tubes are formed with 6 mols or more of Gd III per mol G-actin. No F-actin is formed as judged either by viscometry or by electron microscopy. However, with less than 5.5 mols Gd III per mol G-actin, addition of 0.1 M KCl causes a large increase in the observed viscosity, suggesting that polymerisation into F-actin had occurred. Samples examined at the peak of the viscosity in the electron microscope (Fig. 3) show that the actin has polymerised into F-actin filaments which have aggregated into a loose meshwork. In a few places we can observe a side-by-side arrangement of the filaments. F-actin were observed over the range 0–5.5 mols Gd III per mol actin. Thus the lanthanide ions do not inhibit the formation of F-actin until a certain critical molar ratio is reached.

The actin used in this study migrated as a single band on SDS gel electrophoresis. The formation of actin microcrystals and actin tubes is completely reversible when the Gd III concentration is lowered. There is therefore little doubt that these



structures are composed entirely of actin and that their formation is due to the presence of the lanthanide ions. Furthermore, G-actin after incubation with Gd III followed by removal of the lanthanide will polymerise into F-actin⁸. It is evident that the actin has not been denatured by the lanthanide ions. The absence of F-actin formation together with the observation that neither the microcrystals nor the actin tubes possess the 36-nm axial repeat characteristic of the long helical pitch of F-actin shows that these structures are not composed of F-actin and represent some previously undescribed form. Further structural studies are in progress (M. J. D. and C. G. dos R., in preparation). These modes of actin assembly should provide new insights into the size, shape and molecular interactions of actin subunits.

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Asymmetry in the primary structure of undegraded phytochrome

PHYTOCHROME is the photoreceptor for many light-mediated developmental responses in plants¹. This chromoprotein, considered a dimer of apparently identical 120,000-dalton subunits (undegraded phytochrome)^{2,3}, has often been purified from oats as a monomeric 60,000-dalton degradation product (degraded phytochrome)^{2,3} because of its susceptibility to proteases present in crude extracts³. Degraded phytochrome is relatively resistant to further proteolysis and has almost the same photochemical properties as undegraded phytochrome². Immunochemical evidence⁴ indicates that undegraded phytochrome is asymmetric, possessing extensive primary structure not found in degraded phytochrome. In contrast, recent peptide-map data, obtained by a fluorescence procedure, have led to the conclusion that undegraded phytochrome is symmetric, being composed of two nearly identical halves, each half being equivalent to the 60,000-dalton, photoreversible degradation product⁵. We present here autoradiographic tryptic digest maps of iodinated degraded and undegraded phytochrome from etiolated oat (*Avena sativa* L., cv. Garry) seedlings that offer additional evidence concerning asymmetry in the primary structure of undegraded phytochrome.

Two tryptic digest maps of degraded and five maps of undegraded phytochrome were prepared from the same number

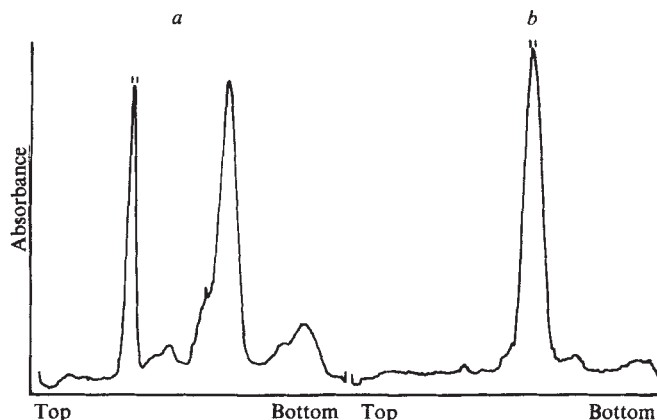


Fig. 1 Absorbance scans of 5 mm diameter, cylindrical SDS-polyacrylamide gels of *a*, an immunoprecipitate of ~10 µg of undegraded phytochrome, with the 120,000-dalton phytochrome subunit (left) and the 50,000-dalton heavy and 25,000-dalton light chains of immunoglobulin G, and *b*, ~10 µg of purified 60,000-dalton degraded phytochrome. The portion of each phytochrome band (central 1 mm) cut out for analysis is indicated by the hash marks. Undegraded phytochrome was partially purified from dark-grown oat seedlings through the first ammonium sulphate fractionation as described previously⁶, and then precipitated with specific antiphytochrome serum. Degraded phytochrome was purified from the same oat cultivar using the method described by Rice *et al.*⁷, except that all buffers before the Bio-Gel P-150 column contained 14 mM 2-mercaptoethanol, and a linear salt gradient elution from brushite was performed before Bio-Gel P-150 chromatography. Both phytochrome preparations were denatured by heating in 1% (w/v) SDS and 0.79 M 2-mercaptoethanol, were electrophoresed through 5% (w/v) polyacrylamide gels with 0.14% (w/v) *N-N'*-methylenebisacrylamide for 5 h at 8 mA per gel, as described by Weber and Osborn⁸, and were stained for protein using Coomassie blue. Mobilities of proteins were compared to standards for size determination (see Fig. 3 in ref. 2).

of different preparations using phytochrome bands cut from SDS-polyacrylamide gels (Fig. 1). The map of degraded phytochrome (Fig. 2*a*) contains all but one of the spots (unlabelled arrow) found on the map of undegraded phytochrome (Fig. 2*b*), which contains nine additional spots of comparable intensity (unlabelled arrows). Replicate maps (not shown) gave the same patterns as in Fig. 2. Unmarked spots seen on one map but not the other are revealed by longer exposures of the same maps and therefore are not unique. The existence of a unique spot on the degraded phytochrome map is reasonable as the endoproteases that produce degraded phytochrome may not have the same specificity as trypsin. At least one terminus of the degraded chromopeptide would then have been cleaved between residues that are recognised by trypsin. The peptide subsequently derived by trypsin degradation would have only part of the primary structure found in the analogous peptide in phytochrome that had not been degraded by endogenous proteases. This peptide could also account for one of the additional spots on the undegraded phytochrome map. The remaining eight additional spots represent peptides not found in degraded phytochrome. Even if our interpretation of which spots are homologous (lettered arrows) is in error, the number of spots on the undegraded phytochrome map that cannot be accounted for on the degraded phytochrome map would still be a minimum of eight. As the number of spots on the map of undegraded phytochrome is almost twice that on the degraded phytochrome map, undegraded phytochrome is apparently composed of only one copy of the degraded molecule and an approximately equal amount of unrelated primary structure.

It has been argued that 40-50 peptides represents the upper limit of resolution of a two-dimensional peptide map¹⁰. As tryptic digestion of even degraded phytochrome yields at least 55-60 peptides⁵, the selectivity of the method used here in

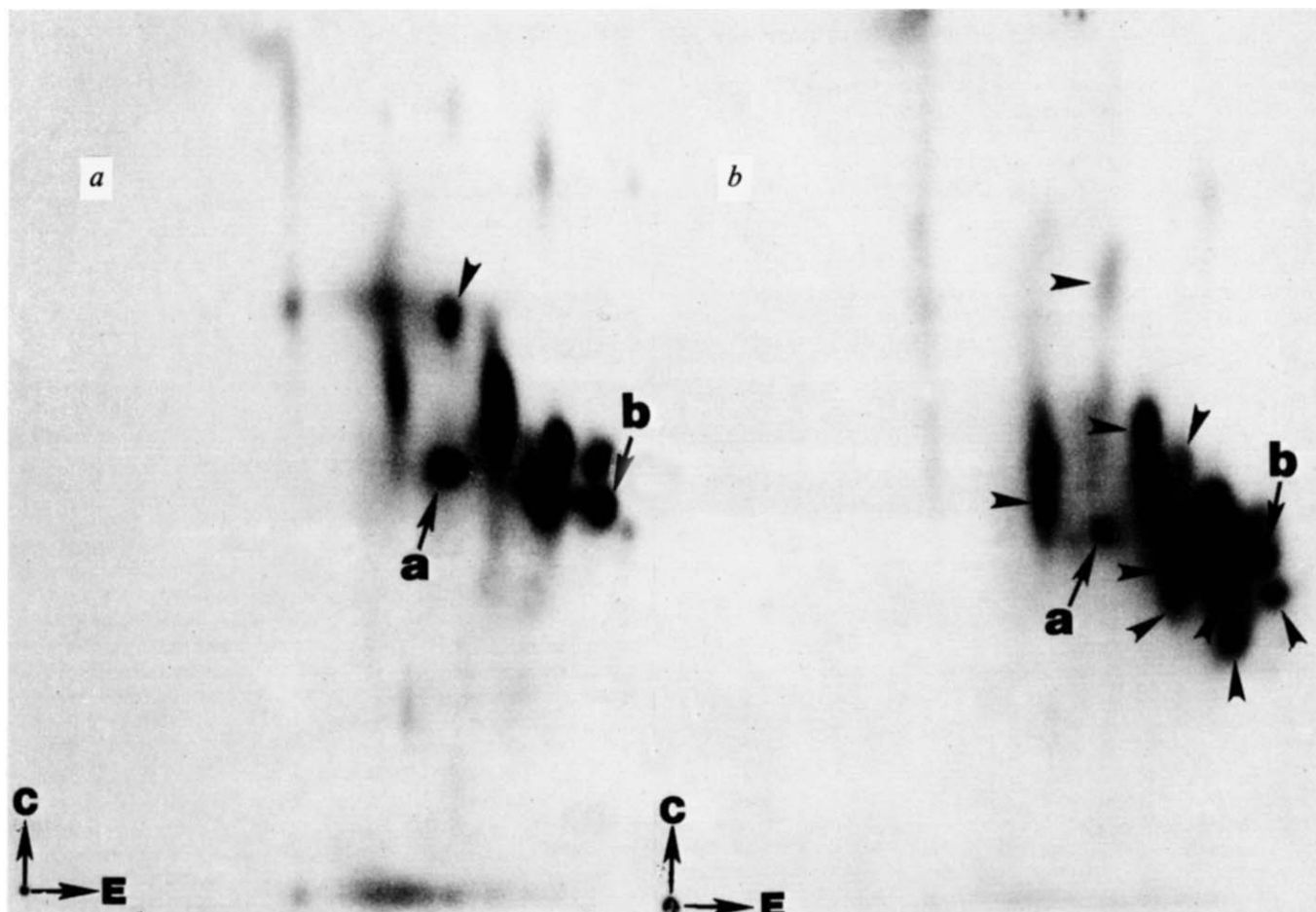


Fig. 2 Autoradiographs of tryptic digest maps of *a*, degraded and *b*, undegraded phytochrome prepared in a single experiment. Lettered arrows indicate corresponding spots; unlettered arrows unique spots. Two dimensional tryptic fingerprinting of ^{125}I -labelled polypeptides was performed essentially as described by Elder *et al.*⁹ Each 1-mm thick gel slice (see Fig. 1) was placed in a siliconised test tube, washed extensively with 25% (v/v) isopropanol and then with 10% (v/v) methanol and dried by vacuum desiccation. The following components were added sequentially to each dried gel slice: 20 μl 0.5 M sodium phosphate buffer (pH 7.5), 300 μCi sodium ^{125}I (NEN, carrier free, 17 Ci mg^{-1}) and 5 μl 1 mg ml^{-1} chloramine T (Sigma). The gel slices were allowed to absorb the liquid for 30–45 min, and then 1 ml of 1 mg ml^{-1} sodium bisulphite was added to terminate the reactions. After 15 min the bisulphite solution was removed. The slices were placed into individual nylon pouches and allowed to dialyse at least 24 h against 4 one-litre changes of 10% (v/v) methanol. Following dialysis each gel slice was removed from its pouch with a spatula, placed in a siliconised tube and dried by vacuum desiccation. To each tube 1 ml of 50 μg ml^{-1} TPCK-trypsin (Worthington) in 50 mM ammonium bicarbonate was added. The gel slices were incubated for 24 h at 37 °C after which each supernatant was removed and lyophilised. Each trypsin-digested sample was dissolved in 20 μl of acetic acid, formic acid and water (15:5:80), and then 5–10 μl of the samples were spotted on 20 $\times$ 20 cm cellulose-coated thin layer plates (Eastman, 13255 cellulose, without fluorescent indicator) for electrophoresis. Electrophoresis was carried out for 1 h at 1 kV in a Savant flat bed electrophoresis apparatus maintained at 2–4 °C. After electrophoresis the plates were dried and the peptides chromatographed in a second dimension using butanol, pyridine, acetic acid and water (32.5:25:5:20). Following chromatography the plates were dried and wrapped in cellophane. Plates were placed over X-Omat R X-ray film (Eastman) so that peptides could be located by autoradiography. Both plates were photographed on a single roll of Kodak Panatomic X film and were printed on paper of identical contrast. Directions of electrophoresis (E) and chromatography (C) are indicated at the origin. Longer exposures of the maps (not shown) indicate that spots on a map that are unmarked (for example, Fig. 2a, intense spot immediately to the left of unmarked arrow and numerous faint spots around main pattern) are also found on the alternate map and therefore are not unique.

visualising primarily only tyrosine-containing peptides, though a potential drawback, is advantageous for our purposes. Residues other than tyrosine are also labelled by this method, but at a lower rate. In addition, the rate of deiodination of these other residues is rapid enough to make it very unlikely that any major spot is due to iodination of a residue other than tyrosine¹¹. As the samples were handled identically, any potential labelling of other amino acids would be identical for both maps.

Amino acid analyses indicate that degraded phytochrome contains 10–18 tyrosyl residues and undegraded about 26 residues^{2,5}. These are sufficient tyrosines to permit critical comparison of the two maps, but few enough to minimise apparent coincidence of similarly migrating, but non-homologous, peptides. The number of spots on the undegraded phytochrome map is smaller than the expected maximum of 26, probably because some peptides contain multiple tyrosines. The

possibility that a peptide is seen in only one map because of the absence of a labelled tyrosine in one case but not the other is precluded because both maps are derived from the same gene products and because these products are labelled after denaturation. Labelling of each sample seems to be complete as the map of degraded phytochrome is completely contained in the map of undegraded phytochrome with the exception of the spot referred to above. Peptides arising from the self-digestion of trypsin¹² are not visualised on the autoradiographs of our maps because trypsin is not added until after residual ^{125}I and chloramine T are removed. Coincidence of a serum protein with the 120,000-dalton phytochrome band is also not a problem as crude serum has no band at this position. Maps prepared from gel slices lacking protein gave totally blank autoradiographs.

The conclusion derived from immunochemical evidence⁴ is the same as that derived from the peptide maps reported here.

Limited proteolysis of undegraded phytochrome yields at least two distinct peptides recognised by antiserum against undegraded but not degraded phytochrome. One of these peptides is ~90,000 daltons as estimated by gel exclusion chromatography. This size, which is probably an overestimate due to non-globular shape, indicates that the peptide, which does not contain a detectable chromophore, represents about one-half of the 120,000-dalton monomer of undegraded phytochrome. As each of the large variety of immunoglobulin Gs in the antiserum against degraded phytochrome recognises a different section of primary structure¹³, it is very unlikely that this '90,000-dalton' peptide has any appreciable primary structure in common with degraded phytochrome.

The data presented here, together with the immunochemical data published earlier⁴, support the same conclusion: that the 120,000-dalton, undegraded phytochrome subunit contains a single copy of the 60,000-dalton, degraded phytochrome monomer and approximately 60,000 daltons of unrelated primary structure. This conclusion conflicts with the earlier interpretation by Stoker *et al.*⁵ of peptide maps (photographs of which were not presented) that visualised all products of tryptic digestion, rather than the limited number examined here.

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C-terminal side of α -helix is more stable than N-terminal side

THE stability of the polypeptide chain of the α -helix has been extensively studied by both experimental and theoretical examination of the transition from the α -helical to random coil conformation of different homopolypeptides^{1,2}. In these studies,

the α -helix has been treated symmetrically along the helical axis. Amino acid residues located at the n th position from the N terminus and the C terminus of the α -helix in a homopolypeptide are assumed to behave in the same manner. However, the stability of the α -helix in the N-terminal region must be different from that in the C-terminal region, due to some extent to the asymmetrical nature of the peptide bond. It has not previously been known whether or not the influence of a polypeptide in random conformation is independent of the side of the α -helix to which the polypeptide is attached. We report here the first experimental evidence for such asymmetrical behaviour.

To detect asymmetry in the α -helix, we prepared block co-polypeptides such that their amino acid compositions were the same but their sequences were reversed with respect to each other; asymmetrical differences in a homopolypeptide are not distinguishable. As previously described³, we synthesised two kinds of block co-polypeptides, (L-Glu)₂₀(L-Ala)₂₀Phe (designated EAF) and (L-Ala)₂₀(L-Glu)₂₀Phe (designated AEF) by a fragment-peptide-coupling technique on a solid support using (BOC-L-Glu)₄ and (BOC-L-Ala)₂. We determined the difference between the α -helical content of each block co-polypeptide in aqueous solution at the same pH, temperature and salt concentration as described below.

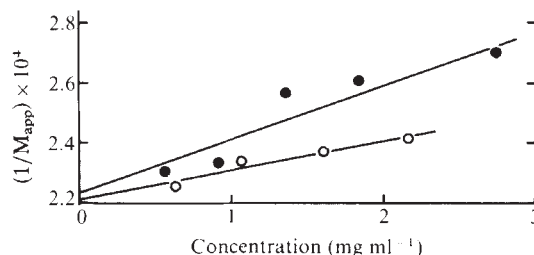


Fig. 1 Results of sedimentation equilibrium experiments for EAF (●) and AEF (○). The experiments were carried out with a Spinco model E analytical ultracentrifuge using the interference optics at 25 °C at 29,500 r.p.m. for 24 h. The block co-polypeptides were dissolved in 0.1 M KF, 20 mM Na-phosphate buffer, pH 8.0 (KF was selected as a neutral salt with a low extinction to 190 nm to measure CD spectra). A partial specific volume of block polypeptides, $\bar{v}$, was taken as 0.63, which is the average weight of partial specific volumes of potassium glutamate (0.57) (refs 12, 13) and alanine (0.74) (ref. 14). The molecular weights (MW) of both polypeptides extrapolated to infinite dilution by the least-squares method were 4,500. M_{app} , apparent molecular weight.

Care was taken to obtain uniform block co-polypeptides. The polypeptides, fractionated by repeated gel filtration on Sephadex G-50, had amino acid compositions of Ala = 21.4, Glu = 21.7, against Phe at the C-terminal end (used as the internal standard) for EAF, and Ala = 19.9, Glu = 20.8 for AEF, in agreement with the calculated values. The presence of any aggregations or micelles could result in artificial differences in the α -helical contents of both block co-polypeptides; micelle formation might occur rather easily for polypeptides containing polyalanine. The materials were monodisperse in solution, as rechromatography on Sephadex G-50 gave only a single peak. This observation was supported by the sedimentation equilibrium experiments shown in Fig. 1. Both polypeptides had the same molecular weight of 4,500, in good agreement with the calculated values from the amino acid compositions. The finding that the slopes against concentration (that is, the second virial coefficients) are different—greater for EAF than for AEF—suggested that the conformations differ from each other in solution. As micelle formation seemed likely to be the most serious problem in this experiment, the circular dichroic (CD) measurements were carried out at various concentrations in the range 0.003–3 mg ml⁻¹. No concentration effect was observed and so further measurements were made at a concentration of 0.1 mg ml⁻¹.

Figure 2 shows the CD spectra of EAF at pH 5.22, 5.56, and 8.53 at 25 °C. The typical CD curve of the α -helix at pH 5.22 has two minima, at 223 nm and 208 nm, and one maximum, at 191 nm; it changes with the increase in pH, indicating that the ionisation of carboxyl groups in L-Glu residues causes the collapse of the α -helix into the random coil conformation. The presence of the isodichroic point at 204 nm implies that the transition occurs between two states, α -helix and random coil. The α -helical spectrum represented by the minimum at 223 nm still occurs at pH 8.53; this may be attributed to the polyalanyl block in the material, for there is no ionisable group in the alanine side chain, whereas the polyglutamyl block takes a random coil conformation at this pH. We examined whether or not the CD spectra of AEF are coincident with those of EAF.

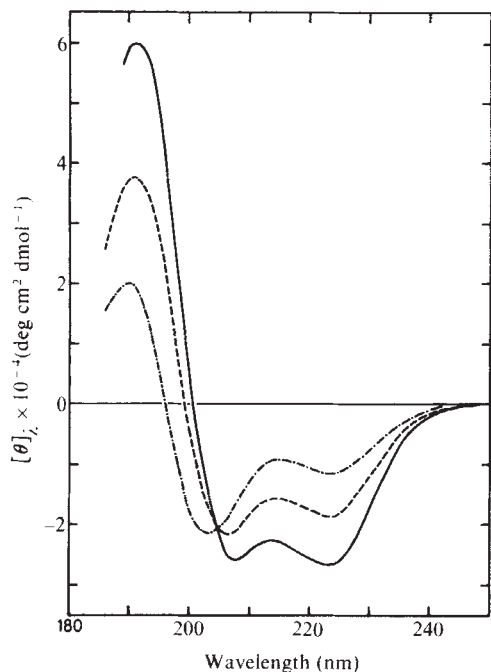


Fig. 2 Circular dichroic spectra for EAF at 25 °C. Solid line, pH 5.22; dashed line, pH 5.56; dashed-dotted line, pH 8.53. CD measurements were carried out with a JASCO J-20 spectropolarimeter using a cell of 1 mm path length. The pH of solutions (in 0.1 M KF) was adjusted by the addition of a small amount of KOH or HClO₄ to the desired values. Concentrations of polypeptides were 0.1 mg ml⁻¹.

Plots of ellipticities at 223 nm, $[\theta]_{223}$ (a measure of α -helical content), for EAF and AEF are shown in Fig. 3 as a function of pH. Apparently, $[\theta]_{223}$ increases with increase in pH and the transition pH of EAF is higher (pH 5.4) than that of AEF (below pH 5.0). The values of $[\theta]_{223}$ become constant above pH 6.5, but are always lower for AEF than for EAF. These results indicate that EAF has a higher α -helical content (32% calculated on the basis that $[\theta]_{223} = -37,000 \text{ deg cm}^2 \text{ dmol}^{-1}$ for the full α -helix, and 900 for the random coil⁴⁻⁶) than AEF (16%) in the same conditions. As the block co-polypeptides are monodisperse in solution (Fig. 1), the stability of the α -helix of the polyalanyl portion must be affected differently by the conformation of the polyglutamyl block and depends on which side the block is connected, the polyalanine helix being more unstable when the charged polyglutamyl block is attached to the C terminus of the helix. Experiments on thermal denaturation of the same materials were consistent with these results, and will be reported in detail elsewhere.

The difference might originate from the Phe residue added to the C terminus as the internal standard, through hydrophobic stabilisation of the α -helix. However, a single additional residue

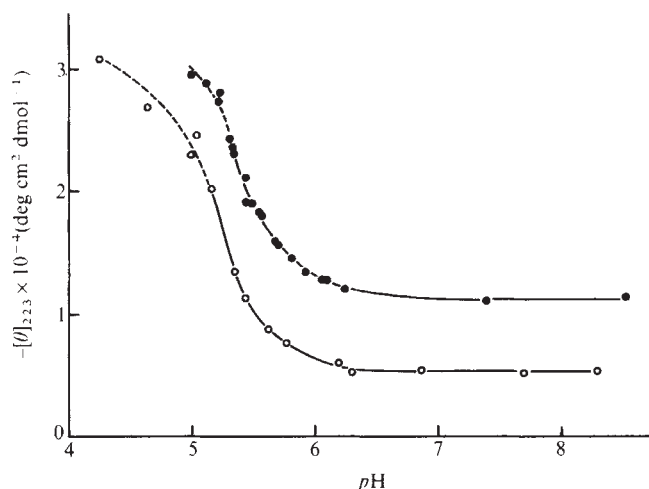


Fig. 3 Ellipticities at 223 nm, $[\theta]_{223}$, plotted against pH for EAF (●) and AEF (○) at 25 °C in 0.1 M KF. The dashed lines represent the region where precipitates appeared. The solutions in this region became turbid with standing, so that accurate measurements were impossible below pH 5.0.

is not sufficient to account for the observed difference of 16% in the helical content. If this were the reason, one extra residue at the C terminus should exert a stabilising effect over 6 to 7 residues in the α -helix. Another possible cause for the asymmetry might be some contribution from the electrostatic interaction of the negative charges on Glu residues with the helix dipole or with peptide dipoles. As the electrostatic interaction in aqueous solution may be weakened by the addition of salts, we examined the effect of salts on the results. The difference observed even at 0.5 M concentration of salts show that the electrostatic effects are not enough to explain the asymmetry, although further detailed examination is required. Therefore, we may conclude that the difference is based on an intrinsic property of the α -helix.

The presence of the asymmetry reported in this study was suggested by simple theoretical calculations on the α -helical conformation^{7,8}. Also, conformational analyses on three-dimensional structures of proteins demonstrate the presence of asymmetrical structures in proteins⁹⁻¹¹; these, however, could be ascribed to heterogeneous alignment of amino acid residues in a protein. A theoretical basis to account quantitatively for this asymmetry of the α -helix remains to be developed.

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matters arising

Palaeogeotherms: implications of disequilibrium in garnet lherzolite xenoliths

FRASER AND LAWLESS¹ have pointed out that, because of the different diffusion mechanisms involved, the two-pyroxene geothermometer^{2,3} and the garnet-orthopyroxene geobarometer^{4,5} commonly used⁶⁻⁸ in the interpretation of the pressure-temperature equilibration conditions and depths of derivation of garnet lherzolite xenoliths in kimberlites may be out of phase and thus generate a spurious apparent geotherm. Whilst this may be the case it should, however, be noted that the lines of constant K for garnet-orthopyroxene equilibria in Fig. 1 of Fraser and Lawless¹ are, in the depth range of interest, effectively sub-parallel to geotherms independently calculated⁹ from heat flow-production studies. This essentially makes it impossible to determine whether geotherms calculated from xenolith data are real or spurious in the way suggested.

Their Fig. 1 thus highlights the critical importance of the temperature estimates to the depths of origin inferred for such xenoliths. Their further discussion, therefore, purporting to demonstrate the different diffusion blocking temperatures for element exchange reactions between various mineral pairs in garnet lherzolite xenoliths is crucial but quite unconvincing and rather misleading.

Fraser and Lawless attempted to test the possibility that diffusion between pyroxenes and garnets is less rapid than between coexisting pyroxenes by comparing the distribution coefficients for $Mg-Fe^{2+}$ exchange between garnet-clinopyroxene and garnet-orthopyroxene pairs with temperatures based on the Ca-Mg exchange between coexisting pyroxenes³. They interpreted the arrays of data points in their Fig. 2 as indicating that $Mg-Fe^{2+}$ exchange between pyroxenes and garnet is effectively blocked below $\sim 1,100^\circ C$ whilst lower temperatures continue to be monitored by Ca-Mg exchange between coexisting pyroxenes. In reaching this conclusion they ignored the fact that the xenoliths concerned have probably been derived from an appreciable depth zone in the mantle and hence may be expected to have equilibrated over a range of pressures as well as temperatures. Furthermore, clearly both $Mg-Fe^{2+}$ exchange between pyroxenes and garnets and Ca-Mg exchange between coexisting pyroxenes depend on pressure as well as temperature, although there are uncertainties as to the magnitudes of the

pressure dependence of these exchange reactions^{2,10-13}. Certainly it is unreasonable for Fraser and Lawless to expect that the xenoliths would plot along lines defining the temperature dependence of the $Mg-Fe^{2+}$ distribution coefficients at effectively one particular pressure. Indeed the experimental evidence of Råheim and Green¹² that $(Mg/Fe^{2+})_{cpx}/(Mg/Fe^{2+})_{gnt}$ decreases with temperature but increases with pressure suggests that the arrays of points on Fig. 2 of Fraser and Lawless may simply reflect the combined pressure/temperature effects on the distribution coefficients involved.

The variation in $(Mg/Fe^{2+})_{cpx}/(Mg/Fe^{2+})_{gnt}$ between garnet-clinopyroxene pairs in different metamorphic environments^{14,15,19} indicates that $Mg-Fe^{2+}$ exchange is normally effective at temperatures well below $1,100^\circ C$. Krogh¹⁶, Råheim and Green¹⁷, for example, have described both prograde and retrograde variations in this distribution coefficient in eclogite assemblages which indicate that above $\sim 700^\circ C$, diffusion rates even in garnets are fast enough to chemically homogenise the minerals. At lower temperatures $Mg-Fe^{2+}$ exchange is not completely blocked but is restricted to the extent that pronounced zoning is produced and retained, especially in the garnets¹⁶.

Thus contrary to the impression given by Fraser and Lawless and to the interpretation which they place on their Fig. 2, petrographic¹⁶⁻¹⁹ and experimental^{2,10-12} evidence indicate that $Mg-Fe^{2+}$ exchange reactions between pyroxenes and garnets probably provide far more sensitive geothermometers below $1,100^\circ C$ than the Ca-Mg exchange between coexisting pyroxenes. However, the magnitude of the pressure effect on the $Mg-Fe^{2+}$ exchange reactions in garnet lherzolite assemblages and reliable estimates of the Fe^{2+}/Fe^{3+} ratios in these minerals¹⁹ must first be determined before one can hope to obtain geologically meaningful temperatures from even these geothermometers and hence calculate valid geotherms.

I thank Dr W. L. Griffin for his helpful comments.

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FRASER AND LAWLESS reply—We completely agree¹ with Carswell that the similarity between the temperature dependence of the garnet-orthopyroxene geobarometer² and likely geothermal gradients makes it difficult to use this reaction to distinguish between the effects of differences in blocking temperatures and temperature-pressure distributions obtained from samples which have equilibrated at different depths along such geothermal gradients.

In the absence of a suitable alternative geobarometer with a different slope it is difficult to distinguish between these two possibilities or indeed some combination of both. It is, however, unsound to make Carswell's *a priori* assumption that the xenoliths in question are derived from different depths and to use such an assumption to require that the nodules show an appreciable effect of pressure on $Fe^{2+}-Mg$ distribution coefficients. This may indeed be the case as may bulk-compositional effects such as we have mentioned¹. However, the observed departure of garnet-pyroxene $Fe^{2+}-Mg$ distribution coefficients from the equilibrium values at 30 kbar is equally consistent with increased departure from equilibrium at lower temperature. The extent of low temperature $Fe^{2+}-Mg$ exchange observed in the eclogitic assemblages of Krogh³ and Råheim and Green⁴ results from equilibration over long periods of geological time and cannot be directly compared with the proposed retrograde effects on garnet lherzolite nodules resulting from relatively rapidly changing temperature-pressure conditions during movement towards the Earth's surface before final eruption of the kimberlite.

These important questions can only be resolved by independent tests. The calibration of an alternative geobarometer with different slope is clearly important. Moreover, the bulk chemical homogeneity of the phases in the xenoliths

should be critically analysed in terms of likely diffusion models and tests should be made of the homogeneity and equilibration of isotopes and refractory trace elements among different mineral phases. Recently published data⁵ show a correlation of pyroxene-garnet Mn/Mg and V/Al ratios with clinopyroxene-orthopyroxene temperatures suggesting equilibration of these trace elements. However, the same authors note grain-by-grain relative inhomogeneities of 25% in Cr and Sc contents of the garnets and similar results have been observed elsewhere⁶. Similarly our work has shown that pyroxene-garnet Cr/Al distribution coefficients are widely scattered for the low temperature group of granular nodules but are more tightly grouped in the case of sheared nodules. Isotopic disequilibrium in garnet and spinel lherzolite nodules has also been reported⁷⁻¹⁰ although it seems that in the case of garnet lherzolites this applies only to sheared nodules, the granular nodules being apparently equilibrated and giving ages close to that of the host kimberlite⁷, despite their dominantly lower temperatures.

There are thus several contradictory pieces of evidence concerning equilibrium in garnet lherzolite and xenoliths and hence uncertainty in their use for the calculation of geothermal gradients. When alternative geobarometers and more isotopic, trace element and kinetic data become available these issues should be more easily resolved.

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Paternal behaviour in fishes: a question of investment, timing or rate?

DAWKINS AND CARLISLE¹ have shown that Trivers' parental investment model² of tetrapod maternal care rests on a fallacy, and suggest that maternal behaviour in tetrapods and paternal behaviour in teleost fishes is a result of the relative timing of gamete release. Like Dawkins³, I view evolution as the selective transfer of information from generation to

generation. However, I believe the fundamental difference between the sexes that leads to exclusive maternal or paternal care is the relative rate at which the two sexes can create gametic copies (spermatogenesis requires less time than oögenesis).

In externally fertilising species, optimal sites for zygote development may constitute a limiting resource for spawning individuals, which selects for site constancy and territorial behaviour. Males are favoured as site-constant individuals because in fishes (as in most vertebrates) males are able to produce new batches of fertile gametes and remate at shorter intervals than females⁴⁻⁸. A male monopolising a site will leave more descendants than a female occupying that site for the same duration. The male can remate, so roving females still spawn at their maximum rate, and their sons will show the site-constant trait. A territorial male's success is limited by the number of females he can attract; hence the sexual adornment in parental male fishes⁹⁻¹¹. Parental behaviour evolves as a consequence of acts by the male that promote survival of the zygotes at his site. Where reproduction is not tied to a site, the female will be favoured as the parent because her potential gain from multiple mating is the lesser.

The relative rate of production of gametes is central to the question of sexual selection and follows directly from Dawkins' property of 'fecundity'³. Questions of efficiency and energy are not important, except in that they influence the rate of reproduction. The model can be used to explain the evolution of anisogamy, substituting rate for the numerical productivity used by Parker, Baker and Smith¹². The present model views the evolution of solitary male or female parental care as a cooperative outcome that allows individuals of each sex to maximise their production of offspring.

I thank Jack P. Hailman, Leigh Hurst and Scott Robinson for helpful discussions.

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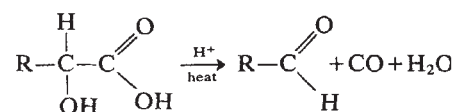
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Acid hydrolysis and α -hydroxy acids in meteorites

THE study by Peltzer and Bada¹ of α -hydroxycarboxylic acids in the Murchison meteorite is of considerable interest because it not only presents a different class of molecules to be looked for in meteorite studies, but also provides a possible method for an evaluation of the ammonia content of the environment in which the synthesis of the organic compounds took place. However, I feel that their analyses of the results leave a room to be filled.

They found less α -hydroxy acids in the acid-hydrolysed water extract fraction than in the unhydrolysed fraction. It is known that α -hydroxy acids decompose to aldehyde and CO when boiled in dilute sulphuric acid, with an overall reaction



As this reaction is known to proceed quite readily, I suspect that this could be the main cause of their observation. Peltzer agreed on this point in his personal communication, saying "the acid-catalysed decomposition of α -hydroxy carboxylic acids probably accounts for the decreased recoveries of the compounds following acid hydrolysis". This in turn would question their conclusion that the hydroxy acids in the meteorite exist primarily in a free or water-hydrolysable state, as their procedure allows whatever α -hydroxy acids are produced through the acid hydrolysis to decompose at the same time to aldehydes. Their conclusion still seems valid, however: Peltzer, in the same personal communication, states that the recoveries of standard compounds at levels equivalent to those found in the meteorite were measured, and that acid hydrolysis recoveries for most hydroxy acids were approximately 10-20% lower than those by water hydrolysis; this extent of the decomposition does not seem large enough to reverse their conclusion.

As the majority of amino acids in meteorites exists in some kind of precursor form and is released only on hydrolysis in 6 M HCl (ref. 2), it would certainly be interesting to study the possible causes for the different states of existence of α -hydroxy acids and of amino acids.

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reviews

Heavy ion reactions

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Nuclear Heavy Ion Reactions. By P. E. Hodgson. Pp.588. (Oxford University Press, Clarendon: Oxford, 1978.) £18.

Our present understanding of the nuclear structure is, to a large extent, based on the information obtained from the analysis of reactions induced by protons, neutrons and light ions, in which the nucleus is moderately excited.

A central feature of the nuclear many-body problem is that the field which confines protons and neutrons is created by the nucleons themselves. This self-confinement is such that the nucleus displays both the degrees of freedom associated with the motion of individual nucleons, as well as collective degrees of freedom where the nucleus reacts as a whole to the external probe. The description of the interplay between the collective and single-particle elementary modes of nuclear excitation constitutes one of the most challenging and exciting problems in the field of many-body physics.

Developments of heavy ion accelerators in recent years have opened the possibility of bringing two heavy ions in contact, thereby making it possible to probe features like the texture of the nuclear surface, and to study nuclear matter under pressures and strains that are only found in the extreme phase of cosmic evolution as, for instance, in the formation of a neutron star.

In his book, Peter E. Hodgson makes a serious attempt at covering most of the subjects of the new and rapidly developing field of heavy ion reactions. Chapter 2 deals with the ion-ion potential. Chapters 3, 5, 6, 7 and 8 deal with the phenomena that can arise in gentle collisions, when the two nuclei barely touch each other (elastic and inelastic scattering and particle transfer reactions, that is, grazing collisions). Chapter 9 treats the aspects of gentle collisions associated with intermediate structure. Chapters 4 and 10 are concerned with the phenomena arising when two ions are brought into intimate contact, and where large amounts of energy and angular momentum are transferred from the relative motion to the internal degrees of freedom, leading to fusion processes and to deep inelastic reactions. In the last half of

chapter 10 the subject of shock waves and relativistic reactions is briefly reviewed. The book starts with a review of the subject of heavy ion reactions (chapter 1) and concludes with an epilogue which plays both the rôle of a résumé to the book and of an educated guess at future trends and lines of development.

The different approaches to all the subjects covered by the book are generously represented, and the referencing, which includes entries up to the first half of 1976, is impressive. These same positive aspects are however also responsible for the fact that this book is a rather confusing catalogue of results more than anything else. In fact, every subject is dealt with by quoting from one or another paper, in many cases not defining even the basic concepts utilised. The conclusions of the authors in question are stated without, apparently, worrying whether they are in contradiction, let alone agreement, with something quoted before or after.

For example, in section 2.2 (p97) it is stated, in connection with the contribution of Satchler to the Nashville conference of 1974: "The double-folding procedure has been used with success to analyse the elastic scattering of alpha particles by nuclei, using standard nucleon-nucleon interactions and phenomenological imaginary potentials. When applied to heavy-ion scattering, however, it is found that the doubly folded potential has to be normalised by a factor around 0.5 to fit the experimental data." Ten pages later we read (p108): "Recent double-folding calculations by Satchler and Love (1976) . . . gave potentials agreeing to within 10–20 per cent with those obtained phenomenologically." It is difficult to understand why Hodgson has neglected to tell us that, as a result of the work of Satchler and Love, Christensen and Winther, and Swiatecki *et al.*, we have now achieved a quantitative understanding of the tail of the ion-ion potential, a prerequisite to any further discussion of heavy ion reactions.

Another example is provided by the discussion on Coulomb-nuclear interference of chapter 5. In the section which goes under the title of DWBA calculations we read (p272): "The Coulomb-nuclear interference in the inelastic scattering of 72.2 MeV ^{11}B

ions by ^{208}Pb . . . [is] given quite well by the distorted-wave theory provided the phase of the nuclear excitation form factor is altered by 5° from that given by the collective model." A couple of pages later, in the section on coupled channel calculations, and when quoting the results of Videbæk *et al.* on the reaction $(^{18}\text{O} + ^{58}\text{Ni}) \rightarrow (^{18}\text{O}(2_1^+) + ^{58}\text{Ni})$ we read that to fit the Coulomb-nuclear interference minimum, multiple step processes have to be invoked. Even so, there remain discrepancies which (p277) "are probably attributable to the neglect of higher-order couplings". Actually, the so-called agreement in the DWBA analysis of the Coulomb-nuclear interference in the reaction $(^{11}\text{B} + ^{208}\text{Pb}) \rightarrow (^{11}\text{B} + ^{208}\text{Pb}(3_1^-))$ and the so-called disagreement in the coupled-channel analysis of the reaction $(^{18}\text{O} + ^{58}\text{Ni}) \rightarrow (^{18}\text{O}(2_1^+) + ^{58}\text{Ni})$ reflect a rather similar open problem, namely that of the role of multiple step processes in the Coulomb-nuclear interference pattern.

Another weakness of the book is that the priorities given to the different subjects discussed do not, in many cases, reflect their physical relevance. For example, in the middle of section 2.4, in which the calculations of the ion-ion potential are discussed, a method to calculate the imaginary potential is taken up without a word of warning. The subject is given one page (p138) and taken up again under the curious subtitle of: "Other calculations of heavy-ion potentials" (p141); here, another page is spent on it. The fact that the calculation of the imaginary part of the ion-ion potential, assuming it is a well-defined problem, is a formidable task implying the reduction of the full set of coupled equations to a local equation for the probability of the system to remain in its ground state, is not even suggested by the author.

One of the most disturbing features of the book is, however, the plain errors it contains—for example, eq. (5.2.3). At first sight it seems we are confronted with the familiar first-order, time-dependent, coupled equations describing the amplitudes associated with the different reaction channels. However, according to the author, these are the equations describing the time evolution of the probabilities associated with

each channel. Because of the central role played by the semiclassical approximation in heavy ion physics, this error has serious consequences, both conceptual as well as practical (for example, the incorrect referencing in p265 to standard computer codes).

On p398 the absolute value of the cross section associated with two-nucleon transfer reactions is discussed. The author should have noted that the cross section increases, due to the inclusion of continuum states in the calculation of the corresponding form factors, not by a factor of 25 but rather by a factor of 2. It is correct that in the reference quoted the value of 25 appears. However, in a later publication the same authors acknowledge an error in their computer code, and the new revised value is given. By the way,

this value of about 2 is similar to the value found by Feng *et al.*, the reference for which is quoted in p422 of this book, also in connection with the absolute cross section of two-particle transfer reactions. It is probably worth noting that an enhancement of the order of 2–4 would be expected from the experience accumulated on this subject in two-particle transfer reactions induced by light projectiles.

According to the cover jacket the book is aimed at graduate students. Let us hope that the excitement which awaits these graduate students entering the field of heavy ion physics, will not be diminished by the rather confusing picture given by this book. □

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Digital image processing

Digital Image Processing and Analysis. Edited by J. C. Simon and A. Rosenfeld. Pp. 513. (Sithoff and Noordhoff: Leiden, The Netherlands, 1977.) \$39.50. *Digital Image Processing.* By W. K. Pratt. Pp. 750. (Wiley: New York and Chichester, UK, 1978.) £20.80.

IN 1976, a NATO Advanced Study Institute was held at Bonas, in south-western France. *Digital Image Processing and Analysis* contains 38 papers delivered at that meeting, printed from authors' typescripts. Conference proceedings are now commonly produced three or four months after submission of the manuscripts and there really is no excuse for allowing nearly two years to elapse, especially in such a fast-moving field as image processing. There is thus not much in this book that has not already appeared in print elsewhere.

The papers are a mixture of introductory surveys by well known scientists and shorter contributions. Of the former, there is little that is not better set out elsewhere, in textbooks such as Pratt's, for example, or Rosenfeld's own volume (for review, see *Nature* 264, 142; 1976); of the other papers, a few contain original material—those on recursive filtering in particular—but most of the material duplicates papers published more rapidly and accessibly in journals.

One redeeming feature of the book is the breadth of coverage. Image enhancement and mensuration are covered as well as pattern recognition, and it is certainly healthy to have these intimately related topics in the same volume. Nevertheless budget-conscious librarians can reasonably regard this volume—unlike several other volumes in the NATO ASI series—as dispensable.

Professor Pratt's volume on *Digital*

Image Processing is a long and detailed textbook, which has grown out of his image processing course given to electrical engineering students in the University of Southern California. The result of several years' work, it is clear, detailed and authoritative, and—a very valuable feature—the author is aware which topics are difficult to grasp and which can be treated in a reasonably summary fashion. Furthermore, it contains sections on colourimetry and the visual aspects of image appreciation as well as the formal mathematics of image processing. There are sections on image coding and on comparatively recent

Magnetospheric physics

Geomagnetic Diagnosis of the Magnetosphere. Physics and Chemistry in Space, Vol. 9. By A. Nishida. Pp.256. (Springer: New York, Heidelberg and Berlin, 1978.)

THE title of this book emphasises the use of geomagnetic data from instruments at ground level for sensing dynamic phenomena occurring far out in the magnetosphere. The value of such observations is also stressed in the preface, and the preface explains that the pursuit of this theme has evolved into an "introduction to magnetospheric physics". In both respects the book is successful, demonstrating how magnetospheric physics can be split with only tolerable damage at a time-scale of about one second. Although processes typified by anomalous diffusion are important for the slow disturbances, it is not necessary to discuss the mechanisms, which involve higher frequencies.

The crudest approximation in plasma physics is hydromagnetics and this provides a useful start. Page 1 is indeed a sudden commencement, but coher-

additions to the field of image processing, such as texture analysis.

The book has the great merit of not only describing the various techniques that are in use but also explaining their purpose and their role in the overall development of this complex subject. As we should expect from a member of the Image Processing Institute, the algebraic aspects of image processing theory are explained clearly and fully but they are not allowed an excessive amount of space.

One criticism can be made, and it is common to most of the recent texts on image processing: the field of electron microscopy, in which the importance of digital image processing has been increasingly recognised during the past few years, is scarcely ever mentioned. This is regrettable, for not only have electron micrographs benefited from some of the techniques described here—originally intended for other types of image—but some quite new procedures have been developed which may well be of interest to students of image processing in general.

This is an extremely well organised and lucid textbook. Despite the author's modest claim that it is intended for use as a course text, many workers already familiar with much of the material will find it useful.

P. W. Hawkes

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ence persists through to the last pulsating chapter, another hydromagnetic success. Much of the intervening chapters is related to reconnection and to particle motion. Dr Nishida has digested a mass of literature on observations and has produced a clear survey, which provides relief after the disjointedness of conference reports. Magnetosphere-ionosphere interactions are included and these sections are of particular relevance to observations using the ground-based incoherent scatter technique; this is timely as a large installation, EISCAT, is now being built.

As an introduction to magnetospheric physics this monograph fills a need; it is splendid for new graduate students or others entering the field. It shows the current rapid progress, being made but warnings such as "the problem of the modelling of the dayside reconnection should not be considered to have been settled" are too weak, and more emphasis might have been given to existing controversies. For the substantial research still needed this book will provide a baseline.

J. W. Dungey

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Dictionary of microbial taxonomy

Dictionary of Microbial Taxonomy. By the late S. T. Cowan. Edited by L. R. Hill. Pp.285. (Cambridge University Press: Cambridge and London, 1978.) £12.50.

TAXONOMISTS, who one might suppose ought to be able to tell us what taxonomy is, seem to agree no more about the meaning than on the derivation of the word. *Taxis* means arrangement: de Caudelle in 1813 coined *taxonomie* and can perhaps by now be forgiven the slip of not spelling it *taxinomie* but he did not explain whether the latter half was from *nomia* (distribution), *nomos* (law) or, mixing Greek with Latin, *nomen* (name). To some, taxonomy means systematics, to others the theoretical study of classification, to others still, nomenclature and the codes which regulate it.

The late S. T. Cowan viewed taxonomy as a scientific art intended to bring order to the often untidy processes of biological classification and nomenclature. Taxonomists he saw as artists—capricious, jealous, argumentative, aggressive—while charitably conceding “Some are good and able to

make a useful contribution to science”. The microbial taxonomist, he felt, should be a systematist dealing equally with classification, nomenclature and identification—“the trinity that is taxonomy”. As a medical and public health bacteriologist, Cowan was often engrossed with practical tasks of identification and he forgave but never forgot the pronouncement of the zoologist, E. Mayr, that identification was the lowest job a systematist did.

Cowan's *Dictionary* contains about 1600 entries, with four introductory chapters, one unfinished, and follows his earlier *Dictionary of Microbial Taxonomic Usage*; the editor, Dr L. R. Hill of the National Collection of Type Cultures, contributes sections on numerical taxonomy. About half of the entries refer to terms or practices in the codes of microbiological nomenclature. These international codes, of Nomenclature of Bacteria, Botanical Nomenclature (covering fungi and algae), and Zoological Nomenclature (including protozoa), with the Rules of Nomenclature of Viruses (a provisional virological code was not ratified) differ in numerous confusing ways. For example, the type of a bacterial species can be and, very sensibly, usually is a living culture maintained in a reference collection, but the type of a fungal or

algal species is generally a dried specimen as required by the Botanical Code. The nomenclatural entries explain the terms, meticulously compare the Codes, where they differ, and give references to the relevant Rules or Articles.

Other entries are technical terms of taxonomy, classification and descriptive microbiology (mostly bacteriology), biographies of taxonomists, addresses of culture collections and advice on scientific authorship; just a few (for example, “chemostat”) have no discoverable relevance to taxonomy.

Many are miniature essays, easy to read and rewarding to the browser, sprinkled with drily entertaining, semi-heretical asides which, for those who did not know Dr Cowan, will convey a flavour of his acrid and sometimes whimsical philosophy. The *Dictionary* is prefaced with an aphorism: “The serious student of nomenclature and the student who takes nomenclature seriously—these are two quite different people”, which reads like a 24-carat hand-turned Cowanism but nicely hints that both types of reader should find the book of value—and they will.

J. E. Smith

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Control of eye movement

The Brain and Regulation of Eye Movement. By A. R. Shakhnovich. Pp. 189. (Plenum: New York, 1978.) \$30.

THERE is an important school of Soviet scientists who have studied eye movement. Matyushkin has described the different muscle fibres of the eye muscles. Yarbus has published his studies on eye movement in the examination of patterns, and Luriya used this technique in the investigation of neurological cases. The author of this book, Dr Shakhnovich, has worked on eye movements, especially in the rabbit, on eye movement in man and on the local cerebral blood flow in man. This book is of interest chiefly for his account of Russian work, for though he is aware of western work, mostly of the 1950s and 1960s, there are important omissions.

It is not, however, easy to follow the accounts of his own work. Although one can accept that local increased activity can and does increase the blood flow in the cerebral cortex so that there are sharply defined local increases and decreases in flow, there

is no successful attempt to relate these changes to the control of eye movement. The illustrations, as Lorrin Riggs remarks in his introduction, are frequently devoid of essential detail of scales and experimental conditions. A little more detail on the technique of recording of eye movement by the Yarbus cap would be helpful, as Yarbus's own account, though admirable, is not readily available here.

In many current problems the author has little help to provide. The function of slow contracture fibres in the eye muscles remains obscure, and the question of vision during saccades is not clarified. As Riggs also remarks, the elegance of the bioengineering approach to the control of eye movement is not exploited in this book.

It is, however, his account of the literature which is unhelpful. “Stimulation” of unknown technique in poorly defined places does not provide a useful basis for study of the controlling mechanisms of eye movement in the midbrain, and it is difficult to follow his argument on the vexed question of reflex responses to stretch in eye muscles.

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Double stars

Double Stars. By W. D. Heintz. Pp. 174. (D. Reidel: Dordrecht, The Netherlands, 1978.) Hardback Dfl.65; \$29; paperback Dfl.30; \$14.

DOUBLE STARS in the sky represent objects of great interest to the astronomer—not only because of their prodigious abundance (more than three-quarters of all stars around us seem to be multiple), but also because a symbiosis of their components provides the best data with which to document our theories on stellar evolution. Observations (positional photometric, spectroscopic) of binary systems have provided almost all we know on the masses and absolute dimensions of the stars, and, in addition, have enabled us to gain more subtle empirical insights into their structure.

In spite of these facts, the students of this important branch of astronomy have so far been served none too well by the existing contemporary literature. For this reason alone the appearance of the book under review is to be welcomed, because in many aspects it usefully fills a noticeable gap. However, this welcome cannot be extended to it without reservation concerning certain parts of its contents, where the latter falls short of the actual needs.

The present reviewer has nothing but praise for the first half of the book concerned with wide (visual or astrometric) binaries—a field in which its author is a recognised expert. That part of its text presents the first comprehensive picture of the field since R. G. Aitken's *Binary Stars* of 1935 (Dover reprint, 1964), brought up-to-date by an account of more recent developments (such as the use of electronic cameras, speckle interferometry, and so on). All this should be very useful to the student and researcher alike—not only by what it describes, but also by many references to the original sources which can guide its user in further reading.

With his account of the close (that is, spectroscopic and photometric) binaries, contained in the second half of the book, the author has been somewhat less fortunate—perhaps because its contents constitute largely a second-hand report which is in many places quite out of date. Thus, on the methodical side (determination of the elements) it does not go really beyond what one could find in Aitken's 1935 classic: Had there been no developments of the subject since that time, would it have been worthwhile to write another book about it? Of course they were; and some of them spectacular; but the author went past them without much notice.

Thus, the reader of Heintz's book will not learn much from it alone on the

effects of distortion (or reflection) on the determination of masses or absolute dimensions of stars constituting close pairs. He will not learn that the process of "rectification" of their light curves represents an empirical make-shift, without theoretical justification, which is apt seriously to falsify the original light message. Hydrodynamical phenomena (gas streams) in close binary systems are still treated in terms of particle mechanics (in which the pressure is ignored); and photometric effects are ascribed to them without appropriate inquiry as to the density of gas which could possess the requisite optical depth.

Basic introduction to pesticides

Pesticides: Preparation and Mode of Action. By R. Cremllyn. Pp. 240. (Wiley: Chichester, UK, and New York, 1978.) £12.

THIS book is aimed specifically at students who are taking first degrees or other qualifications in agriculture or related subjects. It covers, in separate chapters, insecticides, fungicides, herbicides, fumigants, rodenticides, nematocides, molluscicides, and the newer methods of insect control. The basic pattern of these chapters is to start with a brief history of how each particular group of pesticides came to be developed, to give brief details of synthetic routes, and to consider the biological activity.

As with so many agricultural subjects, a wide range of the traditional academic disciplines is involved, from organic chemistry to ecology. The book is obviously written by a chemist: I felt somewhat uneasy with some of the more biological statements. For example, "The nervous system is characteristic of mammals and insects . . ." True, but incomplete, and the uninitiated might wonder which other groups of animals have nervous systems. I am not a professional chemist, but the accounts of synthetic routes seemed to be brief and competent. However, I was less happy with some other aspects of the chemistry. Thus, on p56 DDA is declared to be a metabolite of DDE—as far as I am aware DDA is metabolised only from DDT. Aldrin and dieldrin are both declared (on p64) to be chemically very stable: obviously it depends on how one defines stability, but dieldrin is normally regarded as more stable than aldrin.

These chapters are illustrated with

Moreover, in the last part of the book, the reader is being exposed to various evolutionary 'scenarios' without sufficient caution about their uncertainty or hypothetical nature. This part of the book is certainly not a safe guide for the uninitiated. However, if it succeeds in making him at least interested enough in this field to follow it up by further reading (or work) of his own, it will have satisfied a very useful aim.

Zdenek Kopal

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many structural formulae, which are useful, but identification is inconsistent. Some formulae are named, but many which can be identified by short names bear only a number. This number has to be discovered in the text before the identity of an unknown compound is revealed, which is tedious. There are also 28 black-and-white photographs, all taken by well-known manufacturers of pesticides, and mostly of crops with and without pesticide treatment. They are of somewhat marginal value.

Two introductory chapters discuss broader issues that apply to the whole range of pesticides. One, on physico-chemical factors, discusses briefly the important topics of pesticide design, formulation and selectivity, application and behaviour of pesticides in the environment. The other covers biochemical reactions that are relevant to the mode of action of pesticides: respiration, photosynthesis, conduction of nerve impulses, transport in plants, synthesis of proteins.

The penultimate chapter considers pesticides in the environment, and crystallised some of the reactions I had experienced from the earlier chapters. Facts are often presented in a slightly misleading way, and often key references are absent. References are indeed rather few in number. They are placed at the end of each chapter, and the same standard texts such as Corbett, Hartley and West, Martin and O'Brien recur frequently. This book is of course more up-to-date than its predecessors, but does not replace them.

In summary, I cannot regard this as an authoritative book, but it may be useful for students who do not want to delve too deeply into any aspect of the subject.

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